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EUROPEAN PATENT APPLICATION

⑰ Application number: 89102138.8

⑮ Int. Cl. 4: **C12N 15/00 , C12P 21/02 ,**
A61K 37/02

⑱ Date of filing: 08.02.89

The microorganism(s) has (have) been deposited with American Type Culture Collection and Fermentation Research Institute under numbers CRL-8199 and FERM BP-2223, BP-2226, BP-2225, BP-1409, BP-2224.

⑳ Priority: 08.02.88 JP 27241/88
15.03.88 JP 62841/88

㉑ Date of publication of application:
16.08.89 Bulletin 89/33

㉒ Designated Contracting States:
CH DE ES FR GB IT LI NL SE

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㉖ Human colony-stimulating factors.

㉗ This invention relates to a biologically active recombinant human M-CSF obtained by expressing a human M-CSF gene coding for the amino acid sequence of the formula (1) in its entirety or devoid of a portion thereof; a process for preparing the recombinant human M-CSF; an expression plasmid containing the human M-CSF gene defined above; and a transformant harboring the above expression plasmid. There is also disclosed the use of human M-CSF for the treatment of leukopenia and against infectious diseases.

EP 0 328 061 A2

HUMAN COLONY-STIMULATING FACTORS

The present invention relates to novel human colony-stimulating factors (CSFs), especially recombinant-type CSFs, useful as drugs and to a process for preparing them.

Generally, specific proliferation and differentiation factors are required for the proliferation and differentiation of hematopoietic cells. Various such factors participate in the differentiation and proliferation of hematopoietic cells into various blood cells, such as erythrocytes, granulocytes, macrophages, eosinophils, platelets and lymphocytes (Yasusada Miura, "Blood Stem Cells," Chugai Igaku Sha, 1983). Of these factors, CSFs are known as factors which stimulate the proliferation and differentiation of granulocyte precursor cells and macrophage precursor cells. Known as such CSFs are G-CSF which is specific to the formation of granulocytes, M-CSF which is specific to the formation of macrophages, and GM-CSF which acts to form both granulocytes and macrophages, and further multi-CSF(IL-3) is known which stimulates pluripotent stem cells.

The above-mentioned CSFs, because of their biological activity, are thought to alleviate the leukopenia resulting from cancer chemotherapy and radiotherapy as a common drawback of these therapies. Clinical research on CSFs is conducted from this viewpoint.

The CSFs are also known to have activity to promote the function of leukocytes (Lopez, A.F. et al., J. Immunol., 131, 2983 (1983), Handam, E. et al., same, 122, 1134 (1979) and Vadas, M.A. et al., same, 130, 795 (1983)) and are therefore found effective as drugs for preventing and curing various infectious diseases.

Furthermore, the CSFs are found effective for curing myelogenous leukemia because of its differentiation inducing activity (Metcalf, D. et al., Int. J. Cancer, 30, 773 (1982)).

The activity of CSFs is found, for example, in the cultures of fetal cells, spleen cells, etc. in human urine and in the culture media of various incubated cell lines, and active fractions thereof are separated off and used. However, the CSF of any origin contains large quantities of analogous extraneous substances or the like derived from the starting material and is in itself low in concentration, so that these problems must be solved in preparing the CSF. In respect of homogeneity, yield, ease of operation, etc., therefore, methods still remain to be developed of commercially preparing CSFs as drugs.

We have already established "AGR-ON," a cell line derived from human leukemic T cells, which is capable of producing a large quantity of CSF constitutively in a homogeneous state, and filed for patent application an invention relating to this cell line (Unexamined Japanese Patent Publication SHO 59-169489). We have also developed a process for preparing a CSF from AGR-ON easily in a pure form in a high yield by repeating experiments on the purification of the CSF produced by AGR-ON, further clarified the structural and biochemical characteristics of the resulting CSF, and filed for patent application an invention relating to the CSF as a substance with such features (Unexamined Japanese Patent Publication SHO 62-169799).

The CSF disclosed by the above invention is M-CSF, i.e., a glycoprotein which acts on normal bone marrow cells to promote the differentiation and proliferation of macrophages and which is characterized by the following physicochemical properties. The CSF is termed "AGR-ON-CSF."

a) Molecular weight

33000 to 43000 daltons as determined by SDS polyacrylamide gel electrophoresis under a non-reducing condition, and 23000 to 40000 daltons as determined by gel filtration under a non-reducing condition in the presence of SDS.

b) N-terminal amino acid sequence of the protein portion

The sequence of the following primary structural formula.

Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-Thr

The main object of the present invention is to provide techniques for preparing and supplying human M-CSFs by genetic engineering techniques and the human M-CSFs useful as drugs.

The above object and other features of the invention will become apparent from the following detailed description.

According to the invention, it is provided a biologically active recombinant human M-CSF obtained by

expressing a human M-CSF gen coding for the amino acid sequence of the following formula (1) in its entirety or devoid of a portion thereof.

5 Formula (1):

	1	10
	Met-Thr-Ala-Pro-Gly-Ala-Ala-Gly-Arg-Cys-	
10		20
	Pro-Pro-Thr-Thr-Trp-Leu-Gly-Ser-Leu-Leu-	
		30
	Leu-Leu-Val-Cys-Leu-Leu-Ala-Ser-Arg-Ser-	
		40
15	Ile-Thr-Glu-Glu-Val-Ser-Glu-Tyr-Cys-Ser-	
		50
	His-Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-	
		60
	Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-	
20		70
	Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-	
		80
	Asp-Gln-Glu-Gln-Leu-Lys-Asp-Pro-Val-Cys-	
		90
25	Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-	
		100
	X -Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-	
		110
	Asp-Asn-Thr-Pro-Asn-Ala-Ile-Ala-Ile-Val-	
30		120
	Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys-	
		130
	Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-	
		140
35	Asp-Lys-Ala-Cys-Val-Arg-Thr-Phe-Tyr-Glu-	
		150
	Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-	
		160
	Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-	
40		170
	Asp-Lys-Asp-Trp-Asn-Ile-Phe-Ser-Lys-Asn-	
		180
	Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-	
		190
45	Gln-Asp-Val-Val-Thr-Lys-Pro-Asp-Cys-Asn-	
		200
	Cys-Leu-Tyr-Pro-Lys-Ala-Ile-Pro-Ser-Ser-	
		210
	Asp-Pro-Ala-Ser-Val-Ser-Pro-His-Gln-Pro-	
50		220
	Leu-Ala-Pro-Ser-Met-Ala-Pro-Val-Ala-Gly-	
		230
	Leu-Thr-Trp-Glu-Asp-Ser-Glu-Gly-Thr-Glu-	
		240
55	Gly-Ser-Ser-Leu-Leu-Pro-Gly-Glu-Gln-Pro-	
		250
	Leu-His-Thr-Val-Asp-Pro-Gly-Ser-Ala-Lys-	

260
 Gln-Arg-Pro-Pro-Arg-Ser-Thr-Cys-Gln-Ser-
 270
 Phe-Glu-Pro-Pro-Glu-Thr-Pro-Val-Val-Lys-
 5 280
 Asp-Ser-Thr-Ile-Gly-Gly-Ser-Pro-Gln-Pro-
 290
 Arg-Pro-Ser-Val-Gly-Ala-Phe-Asn-Pro-Gly-
 300
 10 Met-Glu-Asp-Ile-Leu-Asp-Ser-Ala-Met-Gly-
 310
 Thr-Asn-Trp-Val-Pro-Glu-Glu-Ala-Ser-Gly-
 320
 Glu-Ala-Ser-Glu-Ile-Pro-Val-Pro-Gln-Gly-
 15 330
 Thr-Glu-Leu-Ser-Pro-Ser-Arg-Pro-Gly-Gly-
 340
 Gly-Ser-Met-Gln-Thr-Glu-Pro-Ala-Arg-Pro-
 350
 20 Ser-Asn-Phe-Leu-Ser-Ala-Ser-Ser-Pro-Leu-
 360
 Pro-Ala-Ser-Ala-Lys-Gly-Gln-Gln-Pro-Ala-
 370
 Asp-Val-Thr-Gly-Thr-Ala-Leu-Pro-Arg-Val-
 25 380
 Gly-Pro-Val-Arg-Pro-Thr-Gly-Gln-Asp-Trp-
 390
 Asn-His-Thr-Pro-Gln-Lys-Thr-Asp-His-Pro-
 400
 30 Ser-Ala-Leu-Leu-Arg-Asp-Pro-Pro-Glu-Pro-
 410
 Gly-Ser-Pro-Arg-Ile-Ser-Ser-Pro-Arg-Pro-
 420
 Gln-Gly-Leu-Ser-Asn-Pro-Ser-Thr-Leu-Ser-
 430
 35 Ala-Gln-Pro-Gln-Leu-Ser-Arg-Ser-His-Ser-
 440
 Ser-Gly-Ser-Val-Leu-Pro-Leu-Gly-Glu-Leu-
 450
 40 Glu-Gly-Arg-Arg-Ser-Thr-Arg-Asp-Arg-Arg-
 460
 Ser-Pro-Ala-Glu-Pro-Glu-Gly-Gly-Pro-Ala-
 470
 Ser-Glu-Gly-Ala-Ala-Arg-Pro-Leu-Pro-Arg-
 480
 45 Phe-Asn-Ser-Val-Pro-Leu-Thr-Asp-Thr-Gly-
 490
 His-Glu-Arg-Gln-Ser-Glu-Gly-Ser-Ser-Ser-
 500
 50 Pro-Gln-Leu-Gln-Glu-Ser-Val-Phe-His-Leu-

510
 Leu-Val-Pro-Ser-Val-Ile-Leu-Val-Leu-Leu-
 520
 Ala-Val-Gly-Gly-Leu-Leu-Phe-Tyr-Arg-Trp-
 530
 5 Arg-Arg-Arg-Ser-His-Gln-Glu-Pro-Gln-Arg-
 540
 Ala-Asp-Ser-Pro-Leu-Glu-Gln-Pro-Glu-Gly-
 550
 10 Ser-Pro-Leu-Thr-Gln-Asp-Asp-Arg-Gln-Val-
 554
 Glu-Leu-Pro-Val

15 wherein X is Tyr or Asp.

Peptides and amino acids are herein referred to by symbols according to the amino acid nomenclature adopted by IUPAC or by symbols conventionally used in the art. Nucleotide sequences and nucleic acids are also expressed similarly.

20 As already described, the M-CSF of the present invention, because of its biological activity, is useful especially in the field of medicinals as a drug for preventing and curing diseases involving leukopenia, as an auxiliary agent for the bone marrow transplantation, as a drug for preventing and curing various infectious diseases, as an anticancer drug, etc.

The human M-CSFs of the present invention are recombinant-type M-CSFs prepared by genetic engineering techniques and are useful in the above-mentioned fields.

25 The human M-CSF of the invention is prepared utilizing a gene coding for the factor (hereinafter referred to as the "gene of the invention"), i.e., by preparing a recombinant DNA for expressing the gene in host cells, introducing the DNA into host cells for transformation and cultivating the resulting transformant.

A detailed description will be given below of the gene of the invention for use in preparing the human M-CSF of the invention.

30 The gene of the invention is prepared, for example, from human cells having ability to produce M-CSF, more specifically and advantageously from mRNA separated from AGR-ON. The preparation of the gene will hereinafter be described with reference to AGR-ON although the gene can be similarly prepared from other cells. AGR-ON is a human cell line derived from human leukemic T cells and having the characteristics described in Unexamined Japanese Patent Publication SHO 59-169489. The cell line is deposited in 35 American Type Culture Collection (ATCC) as ATCC Deposition No.CRL-8199.

The mRNA is isolated from AGR-ON basically by a usual extraction procedure. Stated more specifically, AGR-ON is cultured, for example, in CEM medium, CMRL-1066 medium, DM-160 medium, Eagle's minimum essential medium (Eagle's MEM), Fisher's medium, F-10 medium, F-12 medium, L-15 medium, NCTC-109 medium, RPMI-1640 medium or the like, with fetal calf serum (FCS) or like serum, or albumin or 40 like serum component added thereto when desired. AGR-ON is inoculated in the medium at a concentration of about 1×10^4 to 1×10^7 cells/ml and is incubated by a usual method, such as culturing in CO₂ incubator, at about 30 to about 40°C, preferably at about 37°C, for 1 to 5 days. When the CSF has been produced and accumulated, the cultured cells are lysed partially or completely with a suitable detergent, such as SDS, NP-40, Triton X100 or deoxycholic acid, or by a homogenizer or by some physical method such as 45 freeze-thaw method. The chromosomal DNA is then sheared to some extent by Polytron (product of Kinematica, Switzerland) or like mixer or a syringe. Proteins are thereafter removed from a nucleic acid fraction for the extraction of total RNA. Generally used for this procedure is, for example, the guanidinium-CsCl ultracentrifugation method (Chirgwin, J.M. et al., Biochemistry, 18, 5294 (1979)).

To prevent the degradation of RNA with RNase, the above method or procedure can be carried out in 50 the presence of RNase inhibitors, such as heparin, polyvinyl sulfate, diethyl pyrocarbonate, vanadyl-ribonucleoside complex.

mRNAs can be separated from the extracted RNA and purified by a column method or batchwise method using, for example, oligo dT-cellulose, poly-U-Sepharose or the like.

55 The mRNA corresponding to the desired M-CSF can be concentrated and identified, for example, by fractionating the mRNAs by sucrose density gradient centrifugation, introducing the fractions into a protein translation system, such as *Xenopus laevis* oocytes, or rabbit reticulocyte lysates, wheat germ extracts or like cell-free system to cause the system to translate the fraction into a protein, and examining the protein for M-CSF activity, whereby the presence of the desired mRNA can be recognized. Instead of the

determination of the M-CSF activity, an immunoassay using an antibody against M-CSF is also available in identifying the desired mRNA.

The purified mRNA thus obtained, which is usually unstable, is converted to a stable cDNA, which is then coupled to a replicon derived from a microorganism for the amplification of the desired gene. The conversion of the mRNA to the cDNA, i.e. the synthesis of the desired gene of the invention can be done generally in the following manner.

Using oligo dT as a primer (which may be dT-tailed vector) and the mRNA as a template, a single-stranded complementary DNA to the mRNA is synthesized therefrom in the presence of dNTP (dATP, dGTP, dCTP or dTTP) with use of a reverse transcriptase. The next step differs as follows depending on whether oligo dT or dT-tailed vector is used.

In the former case, the mRNA used as a template is removed by alkaline hydrolysis, and a double-stranded DNA is synthesized from the single-stranded DNA serving as a template, using a reverse transcriptase or DNA polymerase I. Subsequently, both ends of the double-stranded DNA are treated with exonuclease, a suitable linker DNA or a tail of bases amenable to annealing is attached to each end, and the resulting DNA is inserted into a suitable vector, such as EK-type plasmid vector or λ gt phage vector.

In the latter case, a linear vector-cDNA: mRNA and a linker DNA (frequently used as such is a DNA fragment having a region which can be autonomously replicated in an animal cell and a promoter for the transcription) are annealed into a circular form. The mRNA is thereafter replaced by the DNA strand in the presence of dNTP and also in the presence of both RNaseH and DNA polymerase I to obtain a recombinant plasmid containing cDNA.

The DNA thus obtained is then introduced into a suitable host, such as *Escherichia coli*, *Bacillus subtilis* or *Saccharomyces cerevisiae*, for transformation. The DNA can be introduced into the host, using a usual method, for example, by collecting cells preferably in the logarithmic growth phase, treating the cells with CaCl_2 to make them ready to uptake the DNA. This method can be practiced in the presence of MgCl_2 or RbCl as is generally known to achieve an improved transformation efficiency. The host cells can be converted to spheroplasts or protoplasts before transformation.

From among the transformants thus prepared, the one harboring the plasmid containing cDNA of the desired M-CSF can be selected, for example, by one of the following methods.

(1) Screening with use of synthetic oligonucleotide probe

When the amino acid sequence of the desired protein is wholly or partially has been elucidated (i.e. when a specific sequence of several amino acids in any region of the protein is known), oligonucleotides corresponding to the amino acids are synthesized in view of codon usage of the host cells. Thus synthesized oligonucleotides can be used either as a single or mixed probe. In the latter case, the number of combinations can be reduced by using inosine. The oligonucleotide is labeled with ^{32}P or ^{35}S , and hybridized with nitrocellulose filters having DNAs of the transformants fixed thereto. The desired transformant is selected from the positive hybrids obtained.

(2) Screening of animal cells producing M-CSF

The transformants are incubated to amplify the desired genes, and animal cells are transfected with the amplified genes. (The plasmid to be used in this case is one autonomously replicatable and having a mRNA transcription promotor, or one that will be integrated into an animal cell chromosome.) Thus, the cells are caused to produce proteins coded by the respective genes. The supernatant of each culture of such cells, or the cell extract is assayed for M-CSF activity, or M-CSF is detected using an antibody against M-CSF, whereby the desired transformant having the cDNA specifying M-CSF is selected.

(3) Selection using antibody against M-CSF

cDNA is introduced into vectors which permit the transformant to express a protein. The desired transformant producing M-CSF is detected using an antibody against M-CSF and a second antibody against the antibody.

(4) Use of selective hybridization-translation system

mRNAs from M-CSF producing cells are hybridized with the transformant-derived cDNA immobilized on the nitrocellulose filters, and the desired mRNA corresponding to the cDNA is recovered. The recovered mRNA is subjected to a protein translation system, such as *Xenopus laevis* oocytes, or rabbit reticulocyte lysates, wheat germ extracts or like cell-free system, and is thereby translated into protein. The protein is assayed for M-CSF activity, or is examined for the presence of M-CSF using an antibody against M-CSF to identify the desired transformant.

The DNA coding for M-CSF is obtained from the desired transformant by a known method, for example, by separating a fraction corresponding to the plasmid DNA from the cell, and isolating the cDNA region from the plasmid DNA.

The DNA thus obtained is an example of the gene of the invention coding for a human M-CSF precursor which is defined by the sequence of 372 amino acids shown in Fig. 3 or the sequence of 554 amino acids shown in Fig. 5.

For the substance, obtained by genetic engineering techniques using the gene of the invention, to exhibit the biological activity of human M-CSF, the gene need not always have the above DNA, i.e. the DNA sequence coding for the entire amino acid sequence of the human M-CSF precursor. For example, DNAs coding for portions of the amino acid sequence are also included in the gene of the invention insofar as they permit exhibition of the biological activity of human M-CSF.

The fact that the entire amino acid sequence represented by the formula (1) is not always required for the exhibition of human M-CSF biological activity is apparent also from the fact that the precursor with the sequence of Fig. 3 has the 372nd amino acid (Pro) in the formula (1) at its C terminus, the fact that AGR-ON-CSF has the 35th amino acid (Val) at its N terminus, and the fact that the preparation of AGR-ON-CSF affords a by-product (minor component) which is a biological active M-CSF having the 33rd amino acid (Glu) or the 37th amino acid (Glu) at its N terminus. Further findings as to the similarity in the primary structure between hemopoietins appear to indicate that a naturally occurring M-CSF is present which has the 27th amino acid (Ala) at its N terminus (J.W. Schrader et al., Proc. Natl. Acad. Sci. U.S.A., Vol. 83, pp. 2458-2462 (1986)).

Further as shown in the example concerned and given later, it has been confirmed that the portion of the amino acid sequence of the formula (1) from the 164th amino acid (Trp) to the amino acid at the C terminus is not always necessary for the exhibition of the biological activity of human M-CSF.

Consequently, the gene of the invention is characterized in that the gene specifies a biologically active human M-CSF molecule and has a novel DNA sequence based on the information of the amino acid sequence shown in the formula (1). Stated more specifically, the gene of the present invention includes the DNA coding for the entire amino acid sequence represented by the formula (1), a DNA coding for the amino acid sequence of the formula (1) which is deficient in a portion thereof closer to the N terminus, e.g. in the sequence of the 1st to the 26th, the 1st to the 32nd, the 1st to the 34th or the 1st to the 36th amino acids, and/or in a portion thereof closer to the C terminus, e.g. in the whole or part of the sequence from the 164th amino acid to the C-terminus, and DNAs substantially equivalent to these DNAs.

The gene of the invention can also be prepared by a usual process for the chemical synthesis of nucleic acid, e.g. the phosphite triester process (Nature, 310, 105 (1984)), based on the above information. The present gene can further be prepared from the DNA coding for the polypeptide comprising the sequence of 372 amino acids shown in Fig. 3 or of 554 shown in Fig. 5 by a usual process.

When the gene is to be prepared from the DNA coding for the polypeptide comprising the sequence of 372 or 554 amino acids, usual procedures or means can be employed for the chemical synthesis of portions of the DNA, for the enzymatic treatments for cleaving, removing, adding or ligating DNA strands, and for the isolation and purification, or replication and selection, of the desired DNA. Thus, various known methods are available which are generally employed in the art for genes or DNA strands other than those of the invention. For example, the desired DNA can be isolated and purified by agarose gel electrophoresis, while codons in the nucleic acid sequence can be modified, for example, by site-specific mutagenesis (Proc. Natl. Acad. Sci., 81, 5662-5666 (1984)). The codon to be selected for the desired amino acid is not limited specifically but can be determined in a usual manner in view of the codon usage for the host cell to be utilized, etc.

The DNA sequence of the gene of the invention obtained by the above process can be determined and confirmed, for example, by the Maxam-Gilbert chemical modification method (Meth. Enzym., 65, 499-560 (1980)) or by the dideoxynucleotide chain termination method using M13 phage (Messing, J. and Vieira, J., Gene, 19, 269-276 (1982)).

Thus, human M-CSF can be prepared easily in a large quantity by recombinant DNA techniques using

the gene of the invention.

While it is essential to use the above-specified gene (DNA) of the invention in this process for preparing the human M-CSF, the human M-CSF can be prepared basically by genetic engineering (Molecular Cloning, by T. Maniatis et al., Cold Spring Harbor Laboratory (1982); Science, 224, 1431 (1984); Biochem. Biophys. Res. Comm., 130, 692 (1985); Proc. Natl. Acad. Sci., U.S.A., 80, 5990 (1983); EP Laid-Open Pat. Appln. No.187991).

More specifically, the human M-CSF can be produced by preparing a recombinant DNA which can express the gene of the invention in host cells, introducing the DNA into the host cell and cultivating the transformant.

Useful host cells can be either eukaryotic or prokaryotic cells. The eukaryotic cells include cells of vertebrate animals, yeasts, etc. Generally used as cells of vertebrate animals are, for example, COS cells which are cells of monkey (Y. Gluzman, Cell, 23, 175-182 (1981)), dihydrofolate reductase defective strain of Chinese hamster ovary cell (G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci., U.S.A., 77, 4216-4220 (1980)), etc., while useful cells are not limited to these cells. Useful expression vectors of vertebrate cells are those having a promotor positioned upstream of the gene to be expressed, RNA splicing sites, polyadenylation site, transcription termination sequence, etc. These vectors may further have a replication origin when required. Examples of useful expression vectors include pSV2dhfr having an early promotor of SV40 (S. Sabramani, R. Mulligan and P. Berg, Mol. Cell. Biol., 1, 854-864 (1981)), which is not limitative.

Yeasts are widely used as eukaryotic microorganisms, among which those of the genus *Saccharomyces* are generally usable. Examples of popular expression vectors of yeasts and like eukaryotic microorganisms include PAM82 having a promotor for acid phosphatase gene (A. Miyanojara et al., Proc. Natl. Acad. Sci., U.S.A., 80, 1-5 (1983), etc.

E. coli and *Bacillus subtilis* are generally used as prokaryotic hosts. The present invention employs, for example plasmid vectors capable of replication in the host. To express the gene of the invention in the vector, expression plasmids can be used which have a promotor and SD (Shine-Dalgarno) sequence upstream to the present gene and ATG required for initiating protein synthesis. Widely used as host *E. coli* is *E. coli* K12 strain. pBR322 is a vector which is generally used. However, these are not limitative, and various known strains and vectors are usable. Examples of promotors usable are tryptophan promotor, P_L promotor, lac promotor, 1pp promotor, etc. The gene can be expressed with use of any of these promotors.

For preparing the human M-CSF with use of the gene of the invention, it is desirable, for example, to use COS cells as host cells. The expression vector that can be used in this method is one having SV40 replication origin, capable of autonomously replicating in the COS cell and having a transcription promotor, transcription termination signal, RNA splicing site, etc. For example, when plasmid pcDE is used as shown in the example to follow, the gene of the invention is inserted into the plasmid at the site of restriction enzyme EcoRI positioned downstream from SV40 early promotor, whereby the desired expression plasmid can be obtained. The desired recombinant DNA thus obtained can be introduced into the host cell by a common method. For example, the expression plasmid prepared by inserting the desired gene into the plasmid pcDE can be introduced into COS cells by the DEAE-dextran method or calcium phosphate-DNA coprecipitation method, consequently readily giving the desired transformant.

For preparing the human M-CSF with use of the gene of the invention, another process is desirable in which dihydrofolate reductase defective strain of Chinese hamster ovary cell (CHO-Duk dhfr) is used as the host.

For the preparation of the human M-CSF with use of the gene of the invention, another process is desirable in which a prokaryote such as *E. coli* is used as the host, and a signal peptide is used for secreting and expressing the desired M-CSF outside the inner membrane. Examples of useful signal peptides are various known ones including, for example, Lpp, OmpA, OmpF, PhoE and like outer membrane proteins, and PhoA, Bla, PstS and like periplasm proteins, among which OmpA is especially preferable.

For the preparation of the human M-CSF with use of the gene of the invention, another process is desirable wherein a prokaryote such as *E. coli* is used as the host, and the M-CSF is expressed by a two-cistron system which is a gene expression system comprising two cistrons in sequence. This process yields the desired M-CSF in a large quantity with good stability as accumulated in host cells. This process for preparing the M-CSF of the invention will be described in detail. First, an expression plasmid is prepared which two cistrons, i. e., a gene as the first cistron coding for a suitable polypeptide, and the gene of the invention as the second cistron. It is critical that the plasmid contain upstream from the first cistron a promoter and SD sequence for expressing the gene, and further contain downstream from the first cistron but upstream from the second cistron a synthetic linker which contains an SD sequence for expressing the second cistron, a termination codon for the first cistron and a starting codon for the second cistron, as

arranged in this order.

The gene for use as the first cistron may be a synthetic or natural gene insofar as the gene can be expressed by the host. However, it is noted that the polypeptide encoded in the first cistron is different from the M-CSF of the invention and must subsequently be separated off, so that it is desirable that the polypeptide be hydrophobic and have a molecular weight greatly different from that of the M-CSF. It is therefore desired that the first cistron be one coding for such a polypeptide. Examples of preferred first cistrons are genes and fragments thereof coding for IL-2, IFN- α , - β and - γ , etc. and coding for about 50 to about 100 amino acid residues.

The promoter and SD sequence arranged upstream from the first cistron are those already known. Examples of such promoters are trp promoter, tac promoter, P_L promoter, P_R promoter, 1pp promoter, OmpA promoter, lac promoter and the like, among which trp promoter, P_L promoter and P_R promoter are especially desirable. Examples of useful SD sequences are sequences of 3 to 9 base pairs, such as GGAG and AGGA, capable of forming a hydrogen bond with the 3' terminus of 16S rRNA of prokaryotic cells.

The starting codon and the termination codon present in the synthetic linker to be provided between the first cistron and the second cistron can be any of naturally occurring ones. These codons need not be used each in a complete form, for example, as TGA and ATG, but can be used in a partly overlapping form, for example, as TGATG, TAATG, etc.

The expression plasmid for use in the two-cistron system can be constructed by a usual method (Y. Saito et al., J. Biochem., 101, 1281-1288 (1987); B.S. Schoner et al., Proc. Natl. Acad. Sci., U.S.A., 83, 8506-8510 (1986)). It is especially desirable to prepare the plasmid, for example, by cleaving a plasmid containing the M-CSF gene of the invention with a suitable restriction enzyme, isolating and purifying the fragment containing the M-CSF gene by a usual method, preparing the synthetic linker, for example, by a DNA synthesizer, attaching the linker to the fragment at the upstream side of the M-CSF gene with T4DNA ligase, and incorporating the resulting DNA fragment into a plasmid containing the first cistron and capable of expressing the same, at the proper position. Alternatively, the expression plasmid suitable for the desired two-cistron system can be prepared from the DNA fragment obtained by the above method and containing the second cistron as attached to the synthetic linker, by attaching the first cistron to the upstream end of the fragment, and introducing the resulting DNA fragment into a plasmid having a suitable protein expression system.

The plasmid thus obtained is introduced into suitable host cells for transformation, whereby the desired transformant can be obtained through the two-cistron system. When the transformant is used, the protein relating to the first cistron and the desired M-CSF relating to the second cistron can be expressed individually. The products can be analyzed or identified for example, by SDS-PAGE, Western blotting method or the like, and can be purified as separated from each other by the various methods to be described later.

The desired transformant can be cultivated by a usual method, whereby biologically active human M-CSF is produced and accumulated. The medium to be used for the incubation is one suitably selected from among those usually used for the host cell employed. For incubating the transformant with use of *E. coli* or the like as the host, for example, LB medium, E medium, M9 medium, M63 medium and the like are usable. Various carbon sources, nitrogen sources, inorganic salts, vitamins, natural extracts, physiologically active substances, etc. which are generally known can be added to these media when required. Examples of media usable for COS cells are RPMI-1640 medium, Dulbecco's modified Eagle's MEM, etc. which may be supplemented with fetal calf serum (FCS) or like serum component when so required. The transformant can be incubated under conditions suitable for the growth of host cells, e.g., at a pH of about 5 to about 8, preferably about 7, and at a temperature of about 20 to about 43°C, preferably about 37°C, in the case of *E. coli*.

The M-CSF produced by the transformant intra- or extra-cellularly can be separated off and purified by various separation procedures utilizing the physical or chemical properties of the product. (See for example, "Biological Data Book II," pp. 1175-1259, 1st edition, 1st print, June 23, 1980, published by Kabushiki Kaisha Tokyo Kagakudojin; Biochemistry, Vol. 25, No.25, 8274-8277 (1968); Eur. J. Biochem., 163, 313-321 (1987)). Examples of useful procedures are reconstruction treatment, treatment with use of a usual protein precipitating agent, centrifugation, osmotic shock method, ultrasonication, ultrafiltration, molecular sieve chromatography (gel filtration), adsorption chromatography, ion-exchange chromatography, affinity chromatography, high-performance liquid chromatography (HPLC) and like liquid chromatography, dialysis, and combinations of such procedures.

According to a preferred method of separation, the desired substance is partially purified, from the culture supernatant. First the partial purification is conducted, for example, by a treatment using a salting-out agent such as ammonium sulfate or sodium sulfate and/or ultrafiltration using a flat ultrafiltration

membran , hollow fiber membrane or the like. These treatments are conducted in the same manner as usually done under usual conditions.

The roughly purified product thus obtained is then subjected to adsorption chromatography, affinity chromatography, gel filtration, ion-exchange chromatography, reverse-phase chromatography or the like, or a combination of such procedures to obtain the desired homogeneous substance.

Adsorption chromatography can be conducted using, for example, phenyl-Sepharose, octyl-Sepharose or like resin.

Affinity chromatography can be practiced by chromatography utilizing, for example, ConA-Sepharose, Lentil lectin-Sepharose (product of Pharmacia) or like resin, when so desired.

Gel filtration can be practiced using an agent which is made of dextran gel, polyacrylamide gel, agarose gel, polyacrylamide-agarose gel, cellulose or the like. Examples of useful materials commercially available are Sephadex G type, Sepharose type, Sephacryl type (all products of Pharmacia), Cellulofine (product of Chisso Corporation), Biogel P type, Biogel A type (both products of Bio Rad Lab.), Ultrogel AcA (product of LKB), TSK-GEL SW type (product of Tosoh Corporation), etc.

Ion exchange chromatography can be conducted by chromatography using an anionic ion exchange material, for example with diethylaminoethyl (DEAE) or the like serving as the exchange group.

Reverse-phase chromatography can be conducted using a column which comprises a substrate of silica gel or the like having coupled thereto a ligand such as C₁, C₃ or C₄ alkyl, cyanopropyl or phenyl. More specifically, the chromatography can be conducted with C₄ Hi-Pore Reverse-Phase HPLC column (RP-304, Bio-Rad Laboratories) using acetonitrile, trifluoroacetic acid (TFA), water or the like, or a mixture of such solvents as the eluent.

By the processes described above, the desired M-CSF can be commercially produced with high purity in high yields.

The M-CSFs obtained in this way are in common in that they have CSF activity although slightly different in the N-terminal amino acid sequence, molecular weight, isoelectric point, etc. depending on the kind of gene used for the preparation and kind of system for expressing the gene. Of these M-CSFs, especially desirable are those obtained in the examples given latter.

For use as a drug, the M-CSF of the present invention is formulated into pharmacological compositions containing an effective amount of M-CSF and a usual pharmacologically acceptable nontoxic excipient. The composition is given via a route of administration suited to the form of the composition. Such compositions are, for example, in the form of liquid preparations including solution, suspension, emulsion and the like, which are given usually orally, intravenously, subcutaneously, intracutaneously or intramuscularly. Such forms or methods of administration are not limited specifically; the M-CSF composition can be prepared in various forms usually used and suited, for example, to oral or nonoral administration. The composition can be provided also as a dry preparation which can be reconstituted to a liquid for use by addition of a suitable excipient. While the amount of the composition to be given in any form is not limited specifically but can be determined suitably according to the desired pharmacological effect, the kind of disease, the age and sex of the patient, the degree of disease, etc., the composition is administered usually at a dose of about 0.001 to about 1 mg/kg/day calculated as the amount of the active component, i.e. M-CSF, in terms of protein amount. The daily dose is given singly or dividedly.

The process for preparing the gene of the invention, the process for preparing M-CSF with use of the gene, the features of the invention, etc. will be described in greater detail with reference to the following examples.

The specimens prepared in these examples were assayed for CSF activity by the following method.

Method of determining CSF activity

Fetal calf serum (FCS, 20 ml), 30 ml of α -medium and 20 ml of α -medium of 2-fold concentration are mixed together, and the solution is then maintained at 37°C. A 23.3-ml portion of the solution is admixed with 10 ml of 1% solution of agar (product of Difco Laboratories) already maintained at 50°C to obtain an agar medium, which is then maintained at 37°C.

Bone marrow cells (BMC) collected from the femur of a mouse of BALB/C strain are washed with Hanks' balanced solution twice and thereafter suspended in α -medium to a concentration of 10⁷ cells/ml, and 1 ml of the suspension is added to the agar medium maintained at 37°C. The mixture is thoroughly stirred and then maintained at 37°C. A 0.5-ml portion of the mixture is placed into wells (Tissue Culture Cluster 12, product of Costar Corporation) already containing 50 μ l of a test sample, and the resulting mixture is quickly stirred and then allowed to stand at room temperature. On solidification of the mixture in

ach well, the wells are placed into a CO₂ incubator and incubated at 37° C for 7 days.

The number of colonies thus produced is counted under an optical microscope to provide an index for the CSF activity. When observed morphologically, the colonies formed were mostly macrophag colonies.

The accompanying drawings are as follows.

5 Fig. 1 is a restriction enzyme map of cDNA of λ cM5;

Fig. 2 (Figs. 2-1 to 2-4) shows the nucleotide sequence of the cDNA as determined by the Maxam-Gilbert chemical modification method and the dideoxynucleotide chain termination method using M13 phage;

10 Fig. 3 (Figs. 3-1 and 3-2) shows the primary structure of M-CSF precursor protein coded for by the cDNA;

Fig. 4 (Figs. 4-1 to 4-4) shows the nucleotide sequence of the cDNA of λ cM11;

Fig. 5 (Figs. 5-1 to 5-3) shows the primary structure of M-CSF precursor protein coded for by the λ cM11 cDNA;

Fig. 6 is a diagram showing a procedure for preparing COS cell expression vector pcDE;

15 Fig. 7 is a diagram showing a procedure for preparing M-CSF expression plasmid pcDM*CSF;

Fig. 8 is a diagram showing a procedure for preparing M-CSF expression plasmid pcDM*CSF11-185;

Fig. 9 (Figs. 9-1 to 9-3) is a diagram showing a procedure for preparing M-CSF expression plasmid pIN-III(1pp^P-5)-OmpA-MCSF11-NV151;

20 Fig. 10 shows the amino acid sequence coded for by the M-CSF expression plasmid pIN-III(1pp^P-5)-OmpA-MCSF11-NV151;

Fig. 11 shows the amino acid sequence coded for by M-CSF expression plasmid pIN-III(1pp^P-5)-OmpA-MCSF11-NV143;

Fig. 12 (Figs. 12-1 to 12-2) is a diagram showing a procedure for preparing M-CSF expression plasmid pcDM*CSF11-dhfr;

25 Fig. 13 shows CHO*M-CSF elution profile obtained by DEAE-5PW ion exchange high performance liquid chromatography;

Fig. 14 is a graph showing the result obtained by measuring the isoelectric point of CHO*M-CSF;

Fig. 15 to 20 show the structures of the two-cistron expression systems according to Example 3, (1) to (4);

30 Fig. 21 shows M-CSF elution profile obtained by DEAE-5PW ion exchange high performance liquid chromatography; and

Fig. 22 shows the structures of pcDM*CSF11-dhfrRO, pSV2S-MCSF11-dhfr and pRSVS-MCSF11-dhfr which are M-CSF expression plasmids.

Example 1

Preparation of gene of the invention

(1) Incubation of AGR-ON cells

45 Cultured human T cell line AGR-ON (ATCC deposition No.CRL-8199) was suspended to a concentration of about 10⁵ cells/ml in RPMI-1640 medium (product of Flow-Lab. Inc.) containing 10% newborn calf serum (NCS), 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 100 μ g/ml streptomycin, 100 units/ml of penicillin G, 50 μ g/ml of gentamycin, 5 x 10⁻⁵ M 2-mercaptoethanol and 1 mM glutamine. A one-liter portion of the suspension was incubated in five 200-ml tissue culture flasks (product of Corning) at 37° C for 72 hours.

(2) Extraction of mRNA

55 About 5 x 10⁸ AGR-ON cells obtained by the procedure (1) were dissolved in 50 ml of 4 M guanidine thiocyanate solution [4 M guanidium isothiocyanate, 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 2% Sarkosyl and 140 mM 2-mercaptoethanol], and while heating the solution of 60° C, the DNA was sheared using 50-ml

syringe having 18G injection needle.

To one part by volume of the solution were added 1 part by volume of phenol, 0.5 part by volume of 100 mM sodium acetate (pH 5.2)-10 mM Tris-HCl (pH 7.4)-1 mM EDTA solution, and 1 part by volume of chloroform-isoamyl alcohol (24:1) mixture, as heated to 60 °C. The mixture was shaken in a water bath at 60 °C for 10 minutes and then centrifuged at 4 °C at 3000 r.p.m. for 15 minutes. The aqueous layer was separated off and extracted with phenolchloroform twice and further with chloroform twice. To the combined extract was added cold ethanol in twice the volume of the extract. The mixture was maintained at -20 °C for 60 minutes and centrifuged at 4 °C at 3000 r.p.m. for 20 minutes. The RNA precipitate thus obtained was dissolved in 50 ml of 100 mM Tris-HCl (pH 7.4)-50 mM NaCl-10 mM EDTA-0.2% SDS solution, and proteinase K (product of Merk) was added to the solution to a concentration of 200 µg/ml, followed by reaction at 37 °C for 60 minutes. At 60 °C, the reaction mixture was extracted with phenolchloroform twice and further with chloroform twice. To the combined extract were added 3 M sodium acetate (pH 5.2) in 1/10 the volume of the extract and cold ethanol in twice the volume thereof. The mixture was allowed to stand at -70 °C for 60 minutes and then centrifuged at 4 °C at 3000 r.p.m. for 20 minutes. The RNA precipitate was washed with cold 70% ethanol and thereafter dissolved in TE solution (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA).

In this way, about 5 mg of total RNA was obtained from the 5×10^8 AGR-ON cells.

To obtain mRNA from the total RNA, the RNA was subjected to column chromatography using oligo-(dT)-cellulose (Collaborative Research Inc.). For adsorption, 10 mM Tris-HCl (pH 7.5)-1 mM EDTA-0.5 M NaCl solution was used. The column was washed with the same solution and with 10 mM Tris-HCl (pH 7.5)-1 mM EDTA-0.1 M NaCl solution, and the RNA was then eluted with 10 mM Tris-HCl (pH 7.5)-1 mM EDTA.

The above procedure gave about 110 µg of mRNA.

(3) Preparation of cDNA library

cDNA was prepared from a 5-µg portion of the mRNA obtained by the procedure (2), using cDNA Synthesis System (product of Amersham).

The cDNA (about 0.6 µg) obtained was dried in a vacuum and then dissolved in 20 µl of 50 mM Tris-HCl (pH 7.5)-10 mM EDTA-50 mM DTT-40 µM S-adenosyl-L-methionine solution. After addition of 16 units of EcoRI methylase (product of New England Biolab.), the solution was reacted at 37 °C for 15 minutes.

The reaction mixture was heated at 70 °C for 10 minutes to terminate the reaction and then extracted with phenol-chloroform. To the extract were added 3 M sodium acetate (pH 5.2) in 1/10 the volume of the extract and ethanol in 2.5 times the volume thereof, and the mixture allowed to stand at -70 °C for 15 minutes. The mixture was centrifuged at 4 °C at 15000 r.p.m. for 15 minutes, the DNA precipitate was dissolved in 10 µl of 50 mM Tris-HCl (pH 7.5)-10 mM MgCl₂-10 mM DTT-1 mM ATP-100 µg/ml of EcoRI linker (5'-GGAATTC-3', product of Takara Shuzo Co., Ltd., Japan), 350 units of T4DNA ligase (product of Takara Shuzo Co., Ltd.) was added to the solution, and the mixture was reacted at 14 °C for 16 hours.

The reaction mixture obtained was heated at 70 °C for 10 minutes to terminate the reaction. To the mixture were added 16 µl of 100 mM NaCl-50 mM Tris-HCl (pH 7.5)-7 mM MgCl₂-10 mM DTT solution and 40 units of the restriction enzyme EcoRI (Takara Shuzo Co., Ltd.), and the mixture was maintained at 37 °C for 3 hours for digestion.

To the reaction mixture was added 0.8 µl of 0.5 M EDTA (pH 8.0) to terminate the reaction, and an excess of EcoRI linker was removed by Biogel A50M (product of Bio-Rad Laboratories) column chromatography. To the cDNA fraction was added 1 µg of λgt11DNA (Proclon GT, Promega Biotec.) digested with the restriction enzyme EcoRI and treated with alkaline phosphatase to remove the 5'-phosphate group. Also added to the fraction were 3 M sodium acetate (pH 5.2) in 1/10 the volume of the fraction and ethanol in 2.5 times the volume thereof. The mixture was allowed to stand at -70 °C for 30 minutes and centrifuged at 4 °C at 15000 r.p.m. for 15 minutes. The DNA precipitate was washed with 70% ethanol, then dried in a vacuum and dissolved in 7 µl of water.

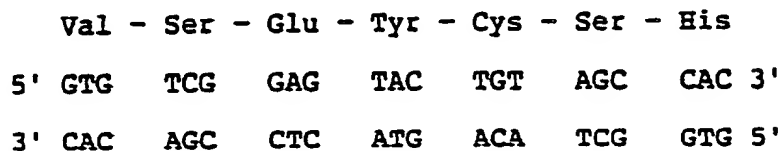
After addition of 2 µl of 500 mM Tris-HCl (pH 7.5)-100 mM MgCl₂, the solution was subsequently maintained at 42 °C for 15 minutes and thereafter cooled to room temperature. To the solution were then added 1 µl of 100 mM DTT, 1 µl of 10 mM ATP and 175 units of T4DNA ligase. The mixture was incubated at 14 °C for 16 hours.

To 10 µl of the reaction mixture was added a λ phage packaging extract (Packagene System, Promega Biotec.), and the mixture was maintained at 22 °C for 2 hours, whereby recombinant phage DNA was packaged in vitro. After addition of 0.5 ml of phage dilution buffer (100 mM NaCl-10 mM Tris-HCl (pH 7.9)-10 mM MgSO₄·7H₂O) and 25 µl of chloroform, the resulting solution was stored at 4 °C.

(4) Screening of cDNA library

(4)-1. Preparation of synthetic probe

The following sequence was used for a synthetic probe based on the information of the amino terminal sequence (27 residues) of AGR-ON-CSF purified from an AGR-ON culture supernatant.



A nucleotide sequence (shown immediate above) complementary to the sequence of the foregoing formula was synthesized by the following procedure in order to use as a probe for selecting a recombinant phage having cDNA coding for M-CSF.

The desired completely protected DNA was synthesized by the solid-phase phosphite triester process (Nature, 310, 105 (1984)) wherein an N,N-dialkylmethylphosphoramidite derivative is used as the condensation unit, using an automatic synthesizer (380A DNA synthesizer, Applied Biosystems Inc.). The completely protected DNA was then treated with 28% of ammonia water at 55°C for 10 hours, whereby the protective groups (i.e. acyl groups on the amino groups of A, G and C) other than the DMTr (dimethoxytrityl) serving as a protective group and attached to the OH at the 5' terminus were removed to give partially protected DNA (to be referred to as "DMTr unit"). The DMTr unit was then purified by reverse-phase high-performance liquid chromatography (HPLC) using a ODS (product of Yamamura Kagaku Kenkyusho Co., Ltd., Japan) column and thereafter treated with 80% acetic acid at room temperature for 20 minutes to obtain a crude oligo nucleotide. The product was further purified by reverse-phase HPLC with the ODS column to prepare the desired oligonucleotide.

The DNA (0.8 µg) obtained was reacted with 18 units of T4 polynucleotide kinase (Takara Shuzo Co., Ltd.) at 37°C for 1 hour in 100 µl of a reaction medium (50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 200 µCi [γ -³²P]-ATP) to label the 5' end of the DNA with ³²P. To separate the labeled DNA from the unreacted [γ -³²P]ATP, the reaction mixture was subjected to gel filtration using Biogel P-30 (Bio-Rad Laboratories). The labeled DNA fractions were pooled and preserved at -20°C. The probe obtained was at least 10⁸ cpm/µg DNA in specific radioactivity.

(4)-2 Plaque hybridization

E. coli Y1090 strain was inoculated in 40 ml of LB medium (10 g/l of Bacto-tryptone, 5 g/l of Bacto-yeast extract and 5 g/l of NaCl) containing 50 µg/ml of ampicillin and 0.2% of maltose and incubated with shaking in a 300-ml flask overnight at 37°C. The culture was centrifuged at 4°C at 3000 r.p.m. for 15 minutes to collect cells. The cell pellets were suspended in 20 ml of SM medium (100 mM NaCl-50 mM Tris-HCl (pH 7.5)-10mM MgSO₄·7H₂O-0.01% gelatin) and stored at 4°C.

To a 0.3 ml portion of the cell suspension was added 0.1 ml of the recombinant phage solution which was obtained by the procedure (3) and diluted to 3.5 x 10⁵ pfu (plaque forming units)/ml with SM medium, and the mixture was then incubated at 37°C for 15 minutes. Subsequently, LB soft agar medium (10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 5 g of NaCl and 7 g/liter of agarose) prewarmed to 47°C was admixed with the mixture, and the resulting mixture was overlaid onto LB agar plate (10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 5 g of NaCl and 15 g/liter of Bacto-agar), and incubated at 42°C overnight. Same procedure was repeated in each of 20 petri dishes.

A nylon filter (BNRG 132, Pall Ultrafine Filtration Corp.) having a diameter of 132 mm was placed over each agar plate having a plaque formed thereon to prepare a replica filter. The agar plate was preserved at 4°C as a master plate.

The filter was treated with 0.5 M NaOH-1.5 M NaCl, then with 0.5 M Tris-HCl (pH 7.5)-1.5 M NaCl and thereafter with 0.3 M NaCl-0.02 M NaH₂PO₄ (pH 7.4)-0.002 M EDTA (pH 7.4), dried in air and baked under vacuum at 80°C for 1 hour.

The baked filter was maintained with gentle shaking at 42°C for 6 hours in 50 ml of 0.75 M NaCl-0.075

M sodium citrate-1.0 mg/ml of Ficoll-1.0 mg/ml of polyvinylpyrrolidone-1.0 mg/ml BSA-10 mM sodium phosphate (pH 6.5)-0.2% SDS-0.1 mg/ml salmon sperm DNA. Subsequently, the filter was placed into the same solution as above having the probe added thereto at a concentration of 10^5 cpm/ml and hybridized at 42°C for 20 hours with gentle shaking.

5 After the hybridization, the filter was withdrawn from the solution, washed with 0.9 M NaCl-0.09 M sodium citrate at room temperature three times and then with the same solution at 56°C for 5 minutes.

The filter was dried in air and thereafter autoradiographed at -70°C for 2 days on an X-ray film (XR5, Eastman Kodak Co.) using intensifying screen.

10 After developing the film, the plaques corresponding to the signal region were scraped off the master plate, and the above procedure is repeated to purify the plaques having a positive signal, whereby positive clone λ cM5 and λ cM11 were eventually isolated.

(5) Structural analysis of the cDNA clone

15

Restriction enzyme analysis of λ cM5 was carried out, and the result is shown in Fig. 1.

As shown in Fig. 1, the cDNA is about 2.5 kb in entire length and has sites of cleavage by BstEII (product of New England Biolab.), NcoI (product of Takara Shuzo Co., Ltd.), SmaI (Takara), KpnI (Takara), EcoRI (Takara) and NdeI (product of New England Biolab.), one cleavage site for each, and two sites of cleavage by each of ScaI, StuI and BamHI (all products of Takara).

20 Next, the nucleotide sequence of the cDNA was determined by the Maxam-Gilbert chemical modification method and the dideoxynucleotide chain termination method using M13 phage. Fig. 2 (Figs. 2-1 to 2-4) shows the result.

Fig. 2 reveals that the region (shown as underlined) of the 227th to the 247th bases from the 5' terminus is complementary to the synthetic probe.

25 When the cDNA of λ cM5 is searched for the longest reading frame, the region of the 125th to the 1240th bases from the 5' terminus was found to be this frame. The amino acid sequence corresponding to the sequence of the 227th to 307th bases and coded by the codons thereof was completely in match with the 27 amino acids at the N terminal region of AGR-ON*CSF. This indicates that the cDNA of λ cM5 is the cDNA coding for an M-CSF precursor protein.

30 Fig. 3 (Figs. 3-1 and 3-2) shows the primary structure of the M-CSF precursor protein predicted from these results.

Fig. 4 (Figs. 4-1 to 4-4) shows the nucleotide sequence of the cDNA of λ cM11 determined in the same manner as above and coding for an M-CSF precursor protein, the predicted primary structure of which is shown in Fig. 5 (Figs. 5-1 to 5-3).

35

Example 2

40

Preparation of recombinant M-CSF (r-MCSF)

45 (1) Preparation of COS cell expression vector pcDE

COS-1 cell used in this example is a cell line which is obtained by transforming monkey renal cell line, CV1, with replication origin (Ori)-deficient SV40, and thereby made to express SV40 early gene and rendered T antigen positive (Cell, 23, 175-182 (1981)).

50 Plasmid PCDV1 (H. Okayama and P. Berg, Mol. Cell. Biol., 3, 280-289 (1983)) was first cleaved with restriction enzyme KpnI, and the protruding 3' end was converted to blunt end by T4 DNA polymerase (product of BRL).

On the other hand, the 5' end of EcoRI linker (5'-GGAATTCC-3') (product of Takara Shuzo Co., Ltd.) was phosphorylated with T4 polynucleotide kinase and ligated to the blunt-ended DNA fragment using T4 DNA ligase. The resultant ligate is cleaved with restriction enzyme EcoRI and further with restriction enzyme HindIII. The reaction product obtained was subjected to agarose gel electrophoresis, whereby EcoRI-HindIII DNA fragment of 2590 bp was isolated and purified.

Plasmid pL1 (H. Okayama and P. Berg, Mol. Cell. Biol., 3, 280-289 (1983)) was cleaved with restriction

enzyme PstI (product of Takara Shuzo Co., Ltd.) and the protruding 3' end was converted to blunt end using T4 DNA polymerase. In the same manner as above, EcoRI linker was ligated to the DNA fragment, the resulting DNA fragment was cleaved with restriction enzyme HindIII, and the reaction product was subjected to agarose gel electrophoresis, whereby EcoRI-HindIII DNA fragment with 580 bp was isolated and purified.

The DNA fragment obtained was ligated to the EcoRI-HindIII DNA fragment previously prepared, using T4 DNA ligase, to afford the desired plasmid pcDE.

Fig. 6 schematically shows the above procedure.

10

(2) Preparation of M-CSF expression plasmid pcDM*CSF

λ cM5 DNA was partially digested with restriction enzyme EcoRI, and size-fractionated on agarose gel to isolate and purify a cDNA fragment (about 2.5 kb) as a partially digested EcoRI fragment.

The COS cell expression vector pcDE obtained by the procedure (1) was cleaved with restriction enzyme EcoRI and ligated to the cDNA fragment prepared above, using T4 DNA ligase, whereby the desired plasmid pcDM*CSF was obtained.

The plasmid thus obtained was introduced into *E. coli* HB101 strain. The desired transformant was selected by the restriction enzyme analysis of the plasmid DNA obtained according to the alkaline lysis method (T. Maniatis et al., Molecular Cloning, pp. 90, Cold Spring Harbor Laboratory (1982)).

Fig. 7 schematically shows the above procedure.

25

(3) Preparation of r-MCSF

COS-1 cells were transfected with the pcDM*CSF prepared by the procedure (2), by the DEAE-dextran method (Proc. Natl. Acad. Sci. U.S.A., Vol. 81, p1070 (1984)) and tested for the production of r-MCSF by the following procedure.

COS-1 cells were first suspended to a concentration of about 2.5×10^5 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). The suspension was placed into Tissue Culture Cluster-6 (product of Costar Corp.) in an amount of 2 ml in each well and incubated overnight at 37°C in a CO₂ incubator.

Subsequently, the medium was removed, the cells were washed with RPMI-1640 medium twice, 1 ml of RPMI-1640 (containing 50 mM Tris-HCl (pH 7.4) and 400 μ g/ml DEAE-dextran (Pharmacia)) containing 10 μ g of pcDM*CSF obtained by the procedure (2) was then added to the cells, and the mixture was allowed to stand in a CO₂ incubator for 4 hours. After removing the medium, the cells were washed twice with RPMI-1640 medium and then incubated for 3 hours with addition of 3 ml of RPMI-1640 medium containing 150 μ M chloroquine (Sigma Chemical Company). The medium was subsequently removed, and the cells were washed with RPMI-1640 medium and thereafter incubated in 10% FCS-containing RPMI-1640 medium at 37°C for 72 hours in a CO₂ incubator.

The culture supernatant and the extract therefrom were assayed for CSF activity at each dilution ratio.

The results are given in Table 1 below, which also shows the results achieved by the same procedure as above using pcDE as a control in place of pcDM*CSF. The assay was conducted in duplicate.

45

50

55

Table 1

Dilution ratio	16	32	64	128
pcDM*CSF				
Supernatant Extract	282 97	274 41	159 0	79 0
pcDE (Control)				
Supernatant Extract	0 0	0 0	0 0	0 0

Each value listed in Table 1 is the number of colonies per plate, the same as in the tables to follow showing the CSF activity.

(4) Preparation of M-CSF expression plasmid pcDM*CSF-185

Using the plasmid pcDM*CSF obtained by the procedure (2), the desired M-CSF expression plasmid pcDM*CSF-185 was prepared according to the site-specific mutagenesis method (Proc. Natl. Acad. Sci., 81, 5662-5666 (1984)) by replacing the codon (AAG) coding for the 186th amino acid (Lys) in Fig. 3 by an termination codon (TAG), the expression plasmid therefore having the 185th amino acid (Thr) in Fig. 3 at its C terminus.

This procedure will be described in detail below.

EcoRI-EcoRI DNA fragment (1.8 kb in size) was excised from plasmid pcDM*CSF and cloned in M13mp11 phage (RF) at EcoRI sites to thereby obtain a single-stranded (ss) DNA (M13-CSF), which was used as a template for mutagenesis.

On the other hand, synthetic oligonucleotide [5'-GTGGTGACCTAGCCTGATT-3' (primer)] was phosphorylated with T4 polynucleotide kinase and then hybridized with the ssDNA (M13-CSF). After annealing, the hybrid was treated with DNA polymerase I (Klenow fragment) and T4 DNA ligase in the presence of dNTP and incubated at 15° C for 18 hours.

The resulting DNA was introduced into JM105 competent cells, and 50 plaques among the plaques formed were inoculated on agar plate and incubated at 37° C for 18 hours. A filter containing the colonies was alkaline-denatured in a usual manner, dried and thereafter baked at 80° C for 2 hours. The filter was prehybridized and thereafter hybridized with ³²P-probe prepared by labeling the 5' end of the above primer with [γ -³²P]ATP at room temperature. The hybridized filter was washed with 6 x SSC (saline sodium citrate) first at room temperature for 10 minutes and then at 56° C for 4 minutes, dried and thereafter subjected to autoradiography at -70° C for 18 hours.

M13-CSF-185 was selected from among the mutant clones and transfected to JM105 to prepare ssDNA and RF DNA.

The exactness of the mutated sequence was confirmed by sequencing the ssDNA by the dideoxynucleotide chain termination method.

The desired plasmid pcDM*CSF-185 was obtained by preparing EcoRI-EcoRI fragment from the RF DNA amplified in the JM105 and inserting the fragment into an expression plasmid in the same manner as in the procedure (3).

Using this plasmid, COS-1 cells were caused to express r-MCSF in the same manner as in the procedure (3). Table 2 below shows the results.

Table 2

Dilution ratio	1	3	9	27	81
pcDM*CSF-185					
Supernatant	288	243	238	173	90

5

10

(5) Preparation of M-CSF expression plasmid pcDM*CSF-177

Using 5'-GCTGAATGATCCAGCCAA-3' as a primer, the desired M-CSF expression plasmid pcDM*CSF-177 was prepared in the same manner as in the procedure (4) by replacing the codon (TGC) coding for the 178th amino acid (Cys) in Fig. 3 by a termination codon (TGA), the expression plasmid therefore having the 177th amino acid (Glu) in Fig. 3 at its C terminus.

Using this plasmid, COS-1 cells were caused to express r-MCSF in the same manner as in the procedure (3). Table 3 below shows the results.

20

Table 3

Dilution ratio	1	3	9	27	81
pcDM*CSF-177					
Supernatant	249	254	166	83	28

25

The results given in Tables 2 and 3 reveal that the desired r-MCSF having M-CSF activity can be expressed by the use of plasmids pcDM*CSF-185 and pcDM*CSF-177 having the gene of the invention.

This indicates that the portion of the amino acid sequence shown in Fig. 3 from the 178th amino acid to the amino acid at the C terminus exerts substantially no influence on the expression of M-CSF molecules having the desired biological activity.

35 (6) Preparation of M-CSF expression plasmid pcDM*CSF11

Plasmid pcDM*CSF11 was obtained by the procedure (2) using λ CM11cDNA in place of λ CM5cDNA. r-MCSF was prepared by the procedure (3) with use of the plasmid, achieving substantially the same results as previously attained.

More specifically stated, λ CM11 DNA was partially digested with restriction enzyme EcoRI, subjected to agarose gel electrophoresis, and the cDNA fragment (about 2.5 kb) was purified as partially digested EcoRI fragment.

The COS cell expression vector pcDE obtained by the procedure (1) was cleaved with restriction enzyme EcoRI and ligated to the cDNA fragment prepared above, using T4 DNA ligase, whereby the desired plasmid pcDM*CSF11 was obtained.

The plasmid thus obtained was introduced into *E. coli* HB101 strain. The desired transformant was selected by the restriction enzyme analysis of its plasmid DNA according to the alkaline lysis method (T. Maniatis et al., Molecular Cloning, pp 90, Cold Spring Harbor Laboratory (1982)).

Further the cDNA (fragment partially digested with EcoRI) of the λ CM11 DNA was inserted into cloning vector pUC19 DNA (product of Takara Shuzo Co., Ltd.) at EcoRI cloning site, giving plasmid pUC MCSF*11. The transformant obtained by introducing the plasmid into *E. coli* HB101 strain has been deposited under the name of *Escherichia coli* HB101/pUC MCSF*11 with deposition number FERM BP-1409 in Fermentation Research Institute Agency of Industrial Science & Technology since July 16, 1987.

55

(7) Preparation of M-CSF expression plasmid pcDM*CSF11-185

Plasmid pcDM CSF11 was digested with restriction enzymes EcoRI and BstEII to isolate and purify

EcoRI-BstEI DNA fragment of about 670 bp.

To the BstEI end of the DNA fragment was ligated a synthetic linker,

5'-GTGACCTGATAAGGATCCG-3'

3'-GACTATTCCTAGGCTTAA-5'

The resultant DNA fragment was then ligated to the above-mentioned plasmid pcDE digested with EcoRI, using T4 DNA ligase to obtain the desired plasmid pcDM*CSF11-185. This plasmid has translation termination codon TGATAA at the site of the synthetic linker and therefore codes for a polypeptide having the 185th amino acid (Thr) in Fig. 5 at its C terminus.

Fig. 8 schematically shows the above process.

Table 4 shows the result achieved in the same manner as in the procedure (3) using the plasmid.

Table 4

Dilution ratio	1	2	4	8
pcDM*CSF11-185				
Supernatant	>200	>200	191	171
Dilution ratio	16	32	64	
pcDM*CSF11-185				
Supernatant	110	84	60	

(8) Preparation of M-CSF expression plasmid pcDM*CSF11-177

Using the plasmid pcDM*CSF11-185 obtained by the procedure (7), the desired M-CSF expression plasmid pcDM*CSF11-177 was prepared by replacing the codon (TGC) coding for the 178th amino acid (Cys) in Fig. 5 by a termination codon (TGA) according to the procedure (5), the expression plasmid thus having the 177th amino acid (Glu) in Fig. 5 at its C terminus. The plasmid was used for expression in the same manner as in the procedure (3), with the result shown in Table 5 below.

Table 5

Dilution ratio	1	2	4	8	16
pcDM*CSF11-177					
Supernatant	192	>200	>200	180	70

(9) Preparation of M-CSF expression plasmid pcDM*CSF11-163

In the same manner as the procedure (4) and using the plasmid pcDM*CSF11-185 obtained by the procedure (7) and 5'-AAGGACTGAAATATTTTC-3' as a primer, the desired M-CSF expression plasmid pcDM*CSF11-163 was prepared by replacing the codon (TGG) coding for the 164th amino acid in Fig. 5 by a termination codon (TGA), the expression plasmid having the 163rd amino acid (Asp) in Fig. 5 at its C terminus.

In the same manner as the procedure (3), the plasmid was caused to express r-MCSF in COS-1 cells. The CSF activity of the resulting supernatant was 12 colonies/plate.

The result indicates that at least the amino acid sequence from the 164th amino acid to the C terminus in the amino acid sequence of the formula (1) exerts no substantial influence on the expression of M-CSF

molecules having the desired biological activity.

(10) Preparation of M-CSF secretory expression plasmid pIN-III(1pp^P-5)-OmpA-MCSF11-NV151

Plasmid pcDM⁺CSF11-185 DNA was digested with EcoRI and BamHI to isolate and purify EcoRI-BamHI DNA fragment (about 680 bp), which was inserted into the EcoRI-BamHI cloning site of secretory expression vector pIN-III-OmpA3 (EMBO J., 3, 2437-2442(1984)) to obtain plasmid pIN-III-OmpA3-MCSF11-185.

The plasmid DNA was digested with XbaI and BamHI to isolate and purify XbaI-BamHI DNA fragment (about 770 bp), which was then inserted into the XbaI-BamHI cloning site of cloning vector pUC118 DNA (product of Takara Shuzo Co., Ltd.) for preparing a single-stranded DNA, whereby plasmid pUC118-OmpA3-MCSF11-185 was obtained.

Using the plasmid, plasmid pUC118-OmpA-MCSF11-NV151 was obtained by such modification in which the codon (GTG) coding for the 35th amino acid (Val) of the polypeptide shown in Fig. 5 can follow the codon (GCC) coding for the C-terminal amino acid (Ala) of OmpA signal peptide, according to the aforementioned method of site-specific mutagenesis.

Stated more specifically, *E. coli* JM105 transformed by plasmid pUC118-OmpA3-MCSF11-185 DNA was infected with helper phage K07 (product of Takara Shuzo Co., Ltd.), followed by incubation at 37° C for 14 hours, to obtain single-stranded (ss) pUC118-OmpA3-MCSF11-185DNA. With use of this DNA as a template for mutagenesis and also using 5'-TACCGTAGCGCAGGCCGTGTCGGAGTACTGTAGC-3' as a primer, the desired plasmid pUC118-OmpA-MCSF11-NV151 was obtained in the same manner as in the procedure (4).

The plasmid DNA was then digested with XbaI and BamHI to isolate and purify XbaI-BamHI DNA fragment (about 540 bp). The DNA fragment thus obtained was inserted into the XbaI-BamHI site of expression vector pIN-III(1pp^P-5)-A3 (Nucleic Acids Res., 13, 3101-3110 (1985)), whereby the desired secretory expression plasmid pIN-III(1pp^P-5)-OmpA-MCSF11-NV151 was prepared for expressing a polypeptide comprising the portion of the sequence shown in Fig. 5 from the 35th amino acid (Val) to the 185th amino acid (Thr).

Figs. 9-1 to 9-3 schematically show the above process. Fig. 10 shows the amino acid sequence encoded in the secretory expression plasmid. In Fig. 10, the underlined portion of the sequence represents the OmpA signal peptide, while the processing site is marked with .

(11) Secretory expression of M-CSF

The pIN-III(1pp^P-5)-OmpA-MCSF11-NV151 obtained by the procedure (10) was introduced into *E. coli* HB101 and JM109 for the secretory production of the polypeptide into the periplasm and the periplasmic fraction having CSF activity was collected.

More specifically stated, the strain harboring the M-CSF secretory expression vector was incubated with shaking in 500 ml of LB medium containing 50 µg/ml of ampicillin at 37° C for 14 hours within a 2-liter flask, followed by centrifugation at room temperature at 5000 r.p.m. for 5 minutes. The cell pellets were suspended in 50 ml of 50 mM Tris-HCl (pH 8.0)-25% sucrose solution, and after addition of 1.25 ml of 250 mM EDTA (pH 8.0), the suspension was maintained at room temperature for 30 minutes with gentle shaking. The suspension was then centrifuged to obtain cell pellets, which were suspended again in 25 ml of ice-cold distilled water. The suspension was cooled in ice for 30 minutes with intermittent gentle shaking and thereafter centrifuged at 4° C at 10000 r.p.m. for 5 minutes to collect a supernatant as the periplasmic fraction.

(12) Preparation of M-CSF secretory expression plasmid, pIN-III(1pp^P-5)-OmpA-MCSF11-NV143

The desired M-CSF secretory expression plasmid, pIN-III(1pp^P-5)-OmpA-MCSF11-NV143, was obtained for expressing a polypeptide comprising the portion of the sequence shown in Fig. 5 from the 35th amino acid (Val) to the 177th amino acid (Glu), by the procedure (10) using pcDM⁺CSF11-177 in place of pcDM⁺CSF11-185.

Fig. 11 shows the amino acid sequence encoded in the plasmid. In Fig. 11, the underlined portion of the sequence represents OmpA signal peptide, and the mark indicates the processing site. In the same

manner as in the procedure (11), the plasmid was introduced into E. coli, from which a supernatant exhibiting CSF activity was obtained as a periplasmic fraction.

5 (13) HPLC of M-CSF

The supernatants obtained by the procedures (6), (7), (8) and (11) were subjected to HPLC under the following conditions.

Column: TSK Gel G3000SW (60 cm x 7.5 mm(diam.), Tosoh Corporation

10 Eluent: PBS⁻-containing 0.005% polyethylene glycol and 0.15 M NaCl.

Flow rate: 0.8 ml/min.

Fraction volume: 0.8 ml/tube/min.

The following results were obtained for the samples with reference to molecular weight markers (glutamate dehydrogenase: 290,000, lactate dehydrogenase: 142,000, enolase: 67,000, adenylate kinase: 32,000, cytochrome C: 12,400).

Sample: Four ml of the culture supernatant obtained by the procedure (6) was concentrated to about 150 μ l by Centricon-10 (product of Amicon)

20 CSF activity was found in a fraction corresponding to 320,000 to 700,000 in molecular weight range (480,000 on the average).

25 Sample: Four ml of the culture supernatant obtained by the procedure (7) was treated in the same manner as above.

CSF activity was found in a fraction corresponding to 66,000 to 86,000 in molecular weight range (76,000 on the average).

30 Sample: Four ml of the culture supernatant obtained by the procedure (8) was treated in the same manner as above.

35 CSF activity was found in a fraction corresponding to 52,000 to 86,000 in molecular weight range (67,000 on the average).

Sample: Two ml of the supernatant of periplasmic fraction obtained by the procedure (11) was concentrated to about 110 μ l in the same manner as above.

40 CSF activity was found at a position corresponding to about 30,000 to 40,000 in molecular weight range (35,000 on the average).

45 (14) SDS-PAGE of M-CSF

Ten ml of the culture supernatant obtained by the procedure (6), (7) or (8) was passed through a column packed with 1 ml of ConA-Sepharose, which was then washed with 5 ml of PBS⁻ and eluted with PBS⁻ (10 ml) containing 0.5 M methyl- α -D-mannoside. A 4-ml portion of the resulting eluate was concentrated with Centricon-10, and the concentrate was used for SDS-PAGE mentioned below.

The periplasmic supernatant as obtained by the procedure (11) was used as another sample therefor.

M-CSF was detected by using a rabbit anti-serum against M-CSF prepared in the conventional manner, after Western blotting of the SDS-PAGE gels.

55 More specifically, SDS-PAGE was carried out according to the method of Laemmli, U. K. (Nature, 277, 680 (1970)) using a mini-vertical slab cell (gel concentration 15%). Western blotting was conducted using Transblot Cell (product of Bio-Rad Laboratories). The nitrocellulose filter having the desired substance transferred there to, was blocked with PBS⁻ containing 1% bovine serum albumin and then reacted with the

rabbit anti-serum against M-CSF first and further with peroxidase-labeled goat anti-rabbit antibody (product of Bio-Rad Laboratories). The resulting nitrocellulose filter was reacted with 4-chloro-1-naphthol solution serving as a color developing substrate for the detection of M-CSF band. Table 6 shows the results.

Table 6

Sample	Molecular weight of detected band (kd)	
	Non-reducing condition	Reducing condition
Supernatant Obtained by (6)	82 150 At least 200 (in stacking gel)	43.5 58 At least 70 (plural)
Supernatant obtained by (7)	40 (faint) 43 45 47	15 (faint) 20.5 26
Supernatant obtained by (8)	44 46 50	19 (faint) 25
Supernatant obtained by (11)	32	17

(15) Preparation of M-CSF expression plasmid pcDM*CSF11-dhfr

Plasmid pSV2-dhfr (Mol. Cell. Biol., 1, 854 (1981)) was cleaved into two fragments with restriction enzymes HindIII and BamHI, and the fragment containing a dihydrofolate reductase (DHFR) gene was isolated and purified.

Next, plasmid pRSV-CAT (Proc. Natl. Acad. Sci. U.S.A., 79, 6777(1981)) was similarly cleaved with HindIII and BamHI, and the fragment containing the LTR (long terminal repeat) portion of Rous sarcoma virus (RSV) was isolated and purified. The two purified fragments were ligated together using T4 DNA ligase.

E. coli HB101 competent cells were transformed by adding the above ligated product. Plasmid DNAs were prepared from ampicillin-resistant colonies and were digested with restriction enzymes. The desired plasmid pRSV-dhfr which showed the predicted restriction enzyme map was obtained.

This plasmid contains the LTR portion of RSV, DHFR gene, intervening sequence and polyadenylation signal derived from SV40, and further a replication origin and ampicillin-resistant gene derived from *E. coli* plasmid pBR322, and expresses DHFR under the control of a promoter contained in the LTR portion.

The plasmid pRSV-dhfr was cleaved into two fragments with NdeI and BamHI, and the fragment containing the DHFR gene was isolated and purified. The DNA fragment obtained was treated with DNA polymerase I (Klenow fragment) to convert the cohesive ends to blunt ends.

On the other hand, the plasmid pcDM*CSF11 obtained by the procedure (6) was cleaved with SalI, and the cleaved ends were similarly converted to the blunt ends by treatment with DNA polymerase I.

The two fragments obtained were ligated together with T4 DNA ligase, and the ligated product was transformed to *E. coli* JM-109 competent cells. Plasmid DNAs were prepared from the ampicillin-resistant colonies obtained, restriction enzyme maps prepared, and the desired pcDM*CSF11-dhfr for expressing both M-CSF and DHFR genes was obtained.

This plasmid contains the following sequences: with respect to M-CSF gene expression, SV40 early promoter (SV40 early), SV40-derived intervening sequence (SV40 intron), M-CSF gene and polyadenylation signal (SV40 polyA), and also with respect to DHFR gene expression, RSV-derived LTR (RSV-LTR), DHFR gene (dhfr), SV40-derived intervening sequence (SV40 intron) and polyadenylation signal (SV40 polyA). The plasmid further contains a replication origin (Ori) and ampicillin-resistant gene (Amp^r) derived from *E. coli* plasmid pBR322. The transformant obtained by introducing the plasmid into *E. coli* HB101 strain has been deposited under the name of *Escherichia coli* HB101/pcDM-CSF11-dhfr with deposit number FERM BP-2224 in Fermentation Research Institute, Agency of Industrial Science & Technology since December 26, 1988.

Figs. 12-1 and 12-2 schematically show the above process.

(16) Preparation of r-MCSF

5

Using the plasmid pcDM*CSF11-dhfr thus obtained, r-MCSF was produced by the procedure (3). The CSF activity of the culture supernatant obtained was 68 in terms of the number of colonies (average) per plate (0 for plasmid pcDE as a control).

10

(17) Expression of M-CSF by pcDM*CSF11-dhfr in CHO cells

Dulbecco's modified minimum essential medium (DMEM medium, product of GIBCO Laboratories) was supplemented with 10% FCS, 10 ml of 100 mM sodium pyruvate, 10 ml of nonessential amino acids, 100,000 units of penicilline G, 200 mg of streptomycin, 80 mg of gentamicin and 53 ml of 7% sodium hydrogencarbonate per liter. To the medium was added chinese hamster ovary dhfr-deficient cells (CHO-DuK dhfr⁻ cells; Proc. Natl. Acad. Sci. U.S.A., 77, 4216(1980)) in the ratio of about 2×10^5 cells per 40 ml of the medium in 75-cm² incubator flask (product of Costar Corp.). The cells were incubated for 6 to 8 days and the supernatant of the culture was collected. CHO-DuK dhfr⁻ cells were also treated with trypsin (product of Flow Lab. Inc.) in the usual manner, suspended in the same DMEM, washed with the same medium and separated from the medium.

The cells were then suspended in a DNA injection solution (0.25 M mannitol-0.1 mM CaCl₂·2H₂O-0.1 mM MgSO₄·7H₂O-0.2 mM Tris-HCl (pH 7.2), Wako Pure Chemical Industries Ltd.).

The M-CSF expression plasmid pcDM*CSF11-dhfr obtained by the procedure (15) was cleaved into linear form with ClaI and thereafter dissolved in the DNA injection solution.

The cell suspension (200 μ l, 2×10^6 cells) and the DNA solution (200 μ l, 30 μ g of DNA) were mixed together, and the mixture was placed into Fusion Chamber CH-2 (product of Shimadzu Seisakusho Ltd.), which was connected to Electric Fusion Device SSH-1 (product of Shimadzu Seisakusho Ltd.). 4.2 kV/cm² pulse was applied to the mixture twice at an interval of 1 second to electrically transfect the DNA into the cells.

The cells having the DNA transfected therein were suspended in 10% dialyzed FCS-1% nonessential amino acid solution (product of Flow Lab. Inc.)-2% HT solution (product of Flow Lab. Inc.)-DMEM medium and incubated on a 24-well plate (product of Costar Corp.).

After incubation for 48 hours, the medium was replaced by a selective medium (DMEM medium containing 10% dialyzed FCS and 1% nonessential amino acid solution). The medium was thereafter replaced by a fresh one every 3 to 4 days.

From among about 200 clones of transformant cells obtained by incubating for 10 to 14 days, 50 clones were selected, treated with trypsin in the usual manner and transferred to a new 24-well plate.

The amplification of the DHFR gene gives resistance to methotrexate (MTX) of high concentration (J.B.C., 253, 1357(1978)), while the gene cotransfected with a DHFR gene are amplified by MTX (J. Mol. Biol., 159, 601 (1982)).

The 50 clones of transformant cells were further incubated in a selective medium containing 20 nM MTX. The proliferated resistant clones were treated with trypsin, then suspended in a selective medium containing 50 nM MTX and incubated. Similarly, the proliferated resistant clones were further incubated in a selective medium containing 100 nM MTX, eventually affording clones which were resistant to 400 nM MTX.

Table 7 below shows the CSF activity of the supernatants of cultures of these resistant clones.

50

55

Table 7

CHO cells Clon No.	Resistant to:	
	MTX 100nM	MTX 400nM
2,3-8	2940	30240
2,3-15	2480	13770
2,3-18	6060	22770
2,3-20	4380	24030
2,3-42	3900	19530

The culture supernatant of CHO cells as prepared by the above procedure was directly used as a sample for SDS-PAGE and analyzed for molecular weight in the same manner as in the procedure (14). Consequently, the M-CSF band was detected at a position corresponding to 90,000 under a non-reducing condition and to 44,000 under a reducing condition.

20

(18) Separation and purification of CHO*M-CSF

Dulbecco's modified minimum essential medium (DMEM medium, product of GIBCO Laboratories) was supplemented with 10% FCS, 10 ml of 100. mM sodium pyruvate, 10 ml of nonessential amino acids, 100,000 units of penicillin G, 200 mg of streptomycin, 80 mg of gentamicin and 53 ml of 7% sodium hydrogen carbonate per liter. To the medium was added CHO cell clone No.2, 3-8 obtained by the procedure (17) in the ratio of about 2×10^5 cells per 40 ml of the medium. The clone was cultivated for 6 to 8 days.

The desired homogeneous human M-CSF (CHO*M-CSF) was isolated from the supernatant of the culture by the following purification steps.

In the following steps, the desired protein was detected by the Western blotting method.

1) ConA-Sepharose affinity chromatography

35

To 650 ml of the CHO cell culture supernatant was added 650 ml of 50 mM sodium borate buffer (pH 8.0) containing 1.0 M NaCl and the mixture was stirred to prepare a sample solution.

The sample solution was applied to a column, 5 x 60 cm, packed with ConA-Sepharose gel (350 ml in volume) and equilibrated with 50 mM sodium borate buffer (pH 8.0) containing 0.5 M NaCl. After thoroughly washing the column with the same buffer, the solution was eluted with the same buffer containing 0.5 M methyl- α -D-mannoside.

CHO*M-CSF was detected only in methyl- α -D-mannoside-eluted fractions.

2) TSKgel DEAE-5PW ion exchange HPLC

45

The active fraction obtained by step 1) was concentrated by ultrafiltration using YM-10 membrane. The concentrate was dialyzed against 50 mM sodium borate buffer (pH 8.0), followed by anion exchange HPLC under the following conditions four times separately.

Column : TSKgel DEAE-5PW (21.5 mm I.D. x 15 cm, product of TOSOH CORPORATION)

Eluent A : 50 mM sodium borate buffer (pH 8.0) containing 5% methanol

Eluent B : 50 mM sodium borate buffer (pH 8.0) containing 1.0 M NaCl and 5% methanol

Flow rate : 3.0 ml/min

Fraction volume: 6 ml/tube/2 min

Gradient programn:

5

10

Time (min)	%B
0	0
10	0
20	10
95	29
100	100
110	100
115	0
130	0

15

Consequently, CHO*M-CSF was eluted in fractions No.34 to No.38 (0.23 to 0.25 M NaCl). Fraction was commenced immediately after the injection of the sample. These fractions were collected and used for the following steps.

3) Gel filtration HPLC

20

The CHO*M-CSF fraction obtained by step 2) was concentrated by ultrafiltration using YM-10 membrane and thereafter subjected to gel filtration HPLC under the following conditions six times separately.

Column : TSKgel G3000SW (60 cm x 21.5 mm I.D., product of TOSOH CORPORATION)

Eluent : 50 mM sodium phosphate buffer (pH 6.8) containing 0.3 M NaCl

Flow rate : 3.0 ml/min

25

Fraction volume: 6 ml/tube/2 min

Consequently, CHO*M-CSF was eluted in fractions No.23 to No.26.

The molecular weight of CHO*M-CSF was estimated to be 114,000 from the eluted position of a standard protein for gel filtration HPLC (product of Oriental Yeast Co., Ltd.)

30

4) Reverse phase HPLC (RP-HPLC)

The CHO*M-CSF fraction obtained by step 3) was concentrated by ultrafiltration using YM-10 membrane and then subjected to RP-HPLC under the following conditions.

35

Column : C₄ Hi-pore reverse phase column (Hi-pore RP-304, product of Bio-Rad Laboratories, 4.6 mm I.D. x 250 mm)

Eluent A : 0.1% TFA

Eluent B : acetonitrile-1% TFA (9:1)

Flow rate : 1 ml/min

40

Fraction volume: 2 ml/tube/2 min

Gradient program:

45

50

Time (min)	%B
0	0
10	0
15	40
85	60
90	100
95	100
100	0
110	0

55

The C₄ RP-HPLC gave fractions No.31 and No.32 (52 to 53% B) containing CHO*M-CSF. These fractions were combined together, neutralized with 200 mM sodium borate buffer (pH 8.0) and thereafter concentrated in vacuo by a centrifugal concentrator (product of TOMY SEIKO CO., LTD.).

5) TSKgel DEAE-5PW ion exchange HPLC

The CHO*M-CSF fraction obtained by step 4) was subjected to TSKgel DEAE-5PW ion exchange HPLC under the following conditions.

- 5 Column : TSKgel DEAE-5PW (7.5 mm I.D. x 75 mm, product of TOSOH CORPORATION)
 Eluent A : 50 mM sodium borate buffer (pH 8.0) containing 5% methanol
 Eluent B : 50 mM sodium borate buffer (pH 8.0) containing 1.0 M NaCl and 5% methanol
 Flow rate : 1.0 ml/min
 Fraction volume: 1 ml/tube/min
 10 Gradient program:

	Time (min)	%B
15	0	0
	7	0
	42	30
	47	100
	52	100
20	57	0
	62	0

Fig. 13 shows the elution profile.

- 25 In the figure, plotted as ordinate are the absorbance of protein at 280 nm (A_{280}) and NaCl concentration (M) vs. the fraction No. as abscissa. The double arrow (\leftrightarrow) shown indicates the eluted position of CHO*M-CSF as detected by Western blotting.

The figure shows that CHO*M-CSF is present and detected in fractions Nos. 39 and 40.

- 30 These fractions were collected, concentrated in vacuo by a centrifugal concentrator and subjected to SDS-PAGE, whereby a single protein band was detected. The band was in coincidence with the band position determined by the Western blotting.

6) N-terminal amino acid sequence of CHO*M-CSF

- 35 The N-terminal amino acid sequence of the CHO*M-CSF thus obtained by step 5) was determined using a gas phase sequencer (Applied Biosystems).

Consequently, it was found that the CHO*M-CSF had the following sequence of ten N-terminal amino acids.

- 40 Glu-Glu-Val-Ser-Glu-Tyr-X'-Ser-His-Met-

It was impossible to identify the amino acid (X') in cycle 7. The cDNA sequence suggests that this amino acid residue is Cys.

7) SDS-PAGE of CHO*M-CSF

- 45 According to the method of Laemmli (Laemmli, U.K., Nature, 277, 680 (1970)), the CHO*M-CSF concentrate obtained by step 5) was dissolved in Laemmli's sample buffer with (2-ME⁺) or without 2-mercaptoethanol (2-ME⁻). The solutions were heat-treated at 95°C for 5 minutes and then subjected to SDS-PAGE under the following conditions.

- 50 Gel : 12% polyacrylamide gel, 1.5 mm in thickness.
 Device : PROTEAN (product of Bio-Rad Laboratories)
 Electrophoresis conditions: constant current of 20 mA for stacking gel, and 30 mA for separating gel.
 Electrophoresed for about 4 hours.
 Staining : Silver Stain Kit (product of Wako Pure Chemical Industries, Ltd.)
 55 Molecular weight marker: Prestained Mark r (product of Bio-Rad Laboratories)

From the result of SDS-PAGE, the molecular weight of CHO*M-CSF was estimated to be about 76000 under nonreducing condition (2-ME⁻) and about 38000 under reducing condition (2-ME⁺). The CSF was electrophoresed as a single band at these positions.

8) Isoelectric point of CHO⁺M-CSF

Using the partially purified sample obtained by step 2), the isoelectric point of CHO⁺M-CSF was determined by the following method.

5 The sample (100 μ l) was applied to a gel (LBK Ampholin PAG plate, pH 3.5-9.5) and electrophoresed at 10 W for 2 hours. The gel was thereafter sliced at a spacing of 5 mm, then placed into 1 ml of 50 mM sodium borate (pH 8.0) and tested for activity after elution for 24 hours. The gel was also sliced at a spacing of 1 cm, then allowed to stand in 500 μ l of distilled water for 24 hours and then subjected to pH determination.

10 The result is shown in Fig. 14, in which the pH and total activity (U) are plotted as ordinate vs. the distance (cm) from the cathode as abscissa.

The results shows that the isoelectric point of CHO⁺M-CSF is pH 4.55.

15 (19) Preparation of M-CSF expression plasmid pcDM⁺CSF11-185-dhfr

The plasmid pcDM⁺CSF11-dhfr prepared by the procedure (15) was cleaved with restriction enzyme XhoI into two fragments, and the fragment containing DHFR gene was isolated and purified.

20 On the other hand, the plasmid pcDM⁺CSF11-185 prepared by the procedure (7) was cleaved with restriction enzyme XhoI into two fragments, and the fragment containing M-CSF11-185cDNA was isolated and purified.

The two kinds of fragments thus obtained were ligated together with T4DNA ligase, and the product was introduced into *E. coli* HB101 competent cells (Takara Shuzo Co., Ltd.) for transformation. Plasmid DNA was prepared from the resulting colonies which were resistant to ampicillin, a restriction enzyme map was then determined, and the desired plasmid pcDM⁺CSF11-185-dhfr was obtained.

25 The plasmid is plasmid pcDM⁺CSF11-dhfr wherein M-CSF11 DNA is converted to M-CSF11-185 DNA. Except this, the plasmid is identical with pcDM⁺CSF11-dhfr. The transformant obtained by introducing the plasmid pcDM⁺CSF11-185-dhfr into *E. coli* HB101 strain has been deposited under the name of *Escherichia coli* HB101/pcDM⁺CSF11-185-dhfr with deposition number FERM BP-2223 in Fermentation Research Institute Agency of Industrial Science & Technology since December 26, 1988.

(20) Expression in CHO cells

35 The plasmid pcDM⁺CSF11-185-dhfr obtained by the procedure (19) was introduced into CHO-Duk dhfr⁻ cells, which were then incubated in the same manner as in the procedure (17) to eventually obtain 50 clones resistant to 400 nM MTX.

Six clones were selected from among these resistant clones and checked for the CSF activity of culture supernatant. Table 8 shows the result.

Table 8

CHO cells Clone No.	CSF activity	
	Active Unit (U/ml)	Number of colonies
4-1	188800	25800
4-7	180500	24600
4-12	338200	46100
4-16	105200	14300
4-20	75700	10300
4-32	296900	40500

55 The CSF activity (U/ml) was calculated from the following equation;

$$\text{CSF activity (U/ml)} = (\text{Number of colonies}) \times (\text{Dilution ratio}) \div 1.5.$$

CHO cells (clones No.4 to No.12) were cultivated in the same manner as in the procedure (18) and the

desired M-CSF in the supernatant was similarly purified (by ConA-Sepharose chromatography, TSKgel DEAE-5PW ion exchange HPLC, gel filtration HPLC, reverse phase HPLC and TSKgel DEAE-5PW HPLC).

The desired protein, i.e., M-CSF, resulting from the purification steps was detected by the Western blotting method.

5 In the same manner as in the procedure (14), the M-CSF fraction obtained by the final purification step was subjected to SDS-PAGE and its molecular weight was determined by the Western blotting method.

The fraction was also electrophoresed for the determination of the isoelectric point in the same manner as in the procedure (18), 8). M-CSF was thereafter detected by staining with Coomassie Brilliant Blue R250 and also by Western blotting with use of 10% methanol-containing 1% acetic acid solution as a transfer
10 buffer.

As a result, the M-CSF had a molecular weight of 42000 and 46000 under nonreducing condition and contained a minor component with a molecular weight of 39000. Under reducing condition, the molecular weight of the main component was 22000 and 27000. A band was also detected at 16000 as a minor component.

15 The isoelectric point (pI) of the M-CSF was 3.5 to 4.6. It was found that the CSF was composed primarily of five kinds of molecules.

(21) Preparation of M-CSF expression plasmid pRSVS-MCSF11-dhfr

20 Plasmid pRSV-S was prepared from the DHFR expression plasmid pRSV-dhfr obtained by the procedure (15), by cleaving the plasmid with restriction enzymes HindIII and BglII to obtain a fragment, which devoided the DHFR gene portion. Both ends of the fragment were converted to SalI ends with SalI linker (Takara Shuzo Co., Ltd.), and ligated with T4 DNA ligase.

25 A DNA fragment including RSV-LTR was isolated from the plasmid pRSV-S and purified, by cleaving the plasmid with restriction enzyme BamHI, making the fragment blunt-ended with DNA polymerase and further cleaving the fragment with restriction enzyme NdeI.

On the other hand, plasmid pSV2-dhfr (Mol. Cell. Biol., 1, 854 (1981)) was cleaved with restriction enzyme EcoRI, and ClaI cleavage site was added with ClaI linker (Takara Shuzo Co., Ltd.) to prepare
30 plasmid pSV2-dhfr-EC. This plasmid was cleaved with restriction enzymes NdeI and PvuII to isolate and purify a DNA fragment containing DHFR gene.

The two DNA fragments thus purified were ligated together with T4DNA ligase to obtain plasmid pRSVS-dhfr.

The plasmid pCDM⁺CSF11-dhfr separately prepared by the procedure (15) was cleaved with restriction
35 enzyme EcoRI to obtain M-CSF gene. Both ends of the fragment were converted to SalI ends using SalI linker (Takara Shuzo Co., Ltd.). The fragment was ligated with the above plasmid pRSVS-dhfr at SalI site using T4DNA ligase. The plasmid was then introduced into *E. coli* HB101 (Takara Shuzo Co., Ltd.).

The resulting M-CSF expression plasmid pRSVS-MCSF11-dhfr was mapped with restriction enzymes to identify the plasmid as the desired plasmid.

40 Fig. 22 shows the structure.

As seen in Fig. 22, the plasmid contains LTR portion of RSV (RSV-LTR) participating in expressing M-CSF, M-CSF gene (M-CSF), SV40-derived intervening sequence (SV40 intron), polyadenylation signal (SV40 polyA), early promoter portion (SV40 early) participating in the expression of DHFR, DHFR gene (dhfr), SV40-derived intervening sequence (SV40 intron) and polyadenylation signal (SV40 polyA). The
45 plasmid further contains replication origin (pBR322 ori) and ampicillin-resistant gene (Ap^r) derived from *E. coli* plasmid pBR322.

(22) Preparation of M-CSF expression plasmid pSV2S-MCSF11-dhfr

50 Plasmid pSV2-S was obtained from the DHFR expression vector pSV2-dhfr-EC prepared by the procedure (21), by cleaving the vector with restriction enzymes HindIII and BglII to remove DHFR gene, converting both ends of the fragment to SalI ends with SalI linker and ligating the ends with T4DNA ligase.

On the other hand, a DNA fragment containing DHFR gene was isolated from plasmid pRSV-dhfr and
55 purified, by cleaving the plasmid with restriction enzyme NdeI, converting both ends of the fragment to BamHI ends with BamHI linker (Takara Shuzo Co., Ltd.) and further cleaving the fragment with restriction enzyme BamHI.

The DNA fragment obtained was ligated at BamHI site of the plasmid pSV2-S with T4DNA ligase to

prepare plasmid pSV2S-dhfr.

M-CSF expression plasmid pcDM⁺CSF11-dhfr was cleaved with restriction enzyme EcoRI, M-CSF gene was separated, both ends of the fragment were converted to Sall ends with Sall linker, the fragment was ligated at Sall site of the plasmid pSV2S-dhfr with T4DNA ligase, and the plasmid was introduced into E. coli HB101.

The plasmid thus obtained was found to be the desired plasmid pSV2S-MCSF11-dhfr by restriction enzyme mapping.

Fig. 22 shows the structure of the plasmid. With reference to Fig. 22, the plasmid contains an early promoter portion (SV40 early) derived from SV40, M-CSF gene (M-CSF), intervening sequence (SV40 intron) derived from SV40, polyadenylation signal (SV40 polyA), LTR portion derived from RSV (RSV-LTR), DHFR gene (dhfr), intervening sequence (SV40 intron) derived from SV40, and polyadenylation signal (SV40 polyA). The plasmid further contains a replication origin (pBV322 ori) and ampicillin-resistant gene (Ap^r) derived from E. coli plasmid pBR322.

(23) Preparation of M-CSF expression plasmid pcDM⁺CSF11-dhfrRO

DHFR expression vector pRSV-dhfr was cleaved with restriction enzyme BamHI, the cleavage site was made to have Sall end with Sall linker, the fragment was further cleaved with restriction enzyme NdeI, and Clal end was formed at the cleave site with Clal linker to obtain plasmid pRSV-dhfr-CS.

This plasmid was cleaved with restriction enzymes Clal and Sall to isolate and purify a DNA fragment containing DHFR gene.

On the other hand, M-CSF expression plasmid pcDM⁺CSF11 was cleaved with restriction enzymes Clal and Sall to isolate and purify a DNA fragment containing M-CSF gene, which was then ligated with the above DNA fragment containing DHFR gene with T4DNA ligase. The resulting fragment was transformed into E. coli HB101. Restriction enzyme mapping of the plasmid consequently obtained indicated that M-CSF gene and DHFR gene therein were opposite in the direction of transcription, revealing that the plasmid was the desired plasmid pcDM⁺CSF11-dhfrRO.

Fig. 22 shows the structure of the plasmid. With reference to this diagram, the plasmid contains an early promoter portion (SV40 early) derived from SV40, intervening sequence (SV intron), M-CSF gene (M-CSF) and polyadenylation signal (SV40 polyA). The plasmid further contains, as arranged in opposite relation to these in transcription direction, LTR portion of RSV (RSV-LTR), DHFR gene (dhfr), intervening sequence derived from SV40 (SV40 intron) and polyadenylation signal (SV40 polyA). The plasmid further contains a replication origin (pBR322 ori) and ampicillin-resistant gene (Ap^r) derived from E. coli plasmid pBR322.

(24) Introduction of M-CSF expression plasmid into CHO cells and expression of M-CSF

The M-CSF expression plasmids prepared by procedures (15), (19), (22) and (23) were introduced into CHO cells for expressing M-CSF.

The host CHO cells were CHO-dhfr-DG44 cells (Somatic Cell and Molecular Genetics, 12(6), 555 (1986)).

The methods included in procedure (17) were used for the introduction of plasmids, incubation of CHO cells and gene amplification with MTX.

The cells having each plasmid introduced therein were incubated in a selective medium containing 400 nM MTX to obtain resistant clones, which were further incubated in the same medium containing 1 μ M MTX. The clone which was grown in the medium was further incubated in the same medium containing 3 μ M MTX to obtain resistant clones.

The resulting clones resistant to 3 μ M MTX were incubated on a 24-well plate (product of Costar). When cells grown confluent, the cells were collected. A one-fifth volume of the resulting cell suspension was freshly inoculated into 1 ml of MTX-free D-MEM medium containing 10% FCS on a 24-well plate, followed by culturing for 6 days and collection of the culture supernatants.

Each supernatant was checked for the production of M-CSF by determining the molecular weight of M-CSF by Western blotting. The clones with high yields of M-CSF were checked for M-CSF activity.

Table 9 shows the results.

Table 9

5	Plasmid	Clone	CSF activity	
			Active Unit (u/ml)	Number of colonies
	pcDM-CSF11-dhfr	5- 8	2.55×10^5	3.48×10^4
10		5- 9	1.83×10^5	2.50×10^4
		5-10	2.27×10^5	3.10×10^4
		5-14	1.05×10^5	1.43×10^4
15		5-15	1.38×10^5	1.88×10^4
		5-17	1.11×10^5	1.51×10^4
20		5-22	1.54×10^5	2.10×10^4
		5-24	1.81×10^5	2.47×10^4
25	pcDM-CSF11-dhfrRO	6- 2	2.35×10^5	3.21×10^4
		6- 8	1.15×10^5	1.57×10^4
		6-14	3.53×10^5	4.81×10^4
30		6-17	2.35×10^5	3.21×10^4
		6-19	2.59×10^5	3.53×10^4
35		6-20	1.65×10^5	2.25×10^4
		6-22	1.61×10^5	2.20×10^4
40		6-24	2.98×10^5	4.06×10^4

45

50

55

Table 9 (continued)

5	Plasmid	Clone	CSF activity	
			Active Unit (u/ml)	Number of colonies
	pSV2S-MCSF11-dhfr	7- 1	0.18×10^5	0.24×10^4
10		7- 2	1.53×10^5	2.09×10^4
		7- 4	4.03×10^5	5.50×10^4
		7-10	2.35×10^5	3.21×10^4
15		7-16	1.49×10^5	2.03×10^4
		7-17	1.15×10^5	1.57×10^4
20		7-20	0.08×10^5	0.11×10^4
		7-21	2.96×10^5	4.04×10^4
		7-23	1.10×10^5	1.50×10^4
25	pRSVS-MCSF11-dhfr	8-11	2.18×10^5	2.97×10^4
		8-21	4.27×10^5	5.82×10^4
30		8-22	1.12×10^5	1.53×10^4
	pcDM*CSF11-185-dhfr	9- 2	7.88×10^5	10.75×10^4
35		9- 7	2.82×10^5	3.85×10^4
		9- 8	9.02×10^5	12.30×10^4
40		9-11	5.62×10^5	7.67×10^4

All M-CSF expression plasmids used expressed M-CSF in the CHO cells.

45 The M-CSF of all samples checked by Western blotting was equivalent in molecular weight to the M-CSF (CHO*M-CSF) obtained from host CHO-Dukdhfr⁻ cells in procedure (17).

Examples 3

50

Preparation of recombinant M-CSF (r-MCSF) by two-cistron systems

55

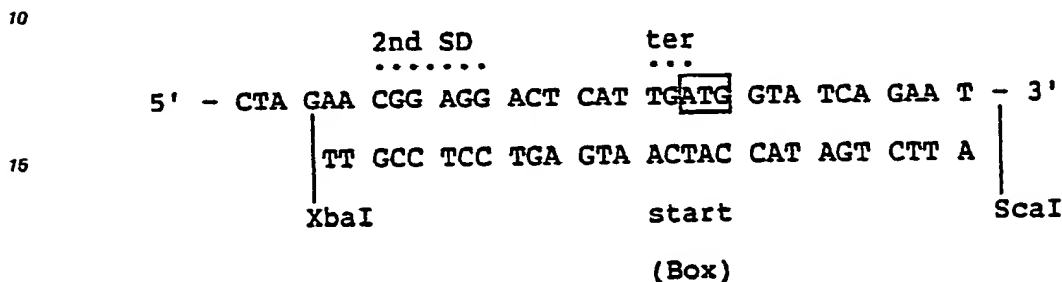
(1) Preparation of M-CSF expression plasmid ptpIL-2X*M*CSF101

The plasmid pcDM*CSF11-185 obtained in Example 2, procedure (7) was digested with restriction

enzymes ScaI and BamHI, and ScaI-BamHI DNA fragment (about 450 bp) was isolated and purified by agarose gel electrophoresis.

The synthetic linker (I) given below was ligated with the cleaved ScaI end of the DNA fragment using T4DNA ligase to obtain XbaI-BamHI DNA fragment (about 480 bp) having XbaI cleavage site toward the ScaI end.

Synthetic linker (I):



The DNA fragment obtained was inserted into XbaI-BamHI cleavage site of human IL-2 expression plasmid ptrpIL-2D8Δ (see Unexamined Japanese Patent Publication SHO 63-12958), giving the desired plasmid ptrpIL-2X*M*CSF101. The transformant obtained by introducing the plasmid into *E. coli* HB101 strain has been deposited under the name of *Escherichia coli* HB101/ptrpIL-2X-M-CSF101 with deposition number FERM BP-2226 in Fermentation Research Institute Agency of Industrial Science & Technology since December 28, 1988.

This plasmid has two polypeptides coded for within a transcription unit governed by *E. coli* tryptophan promoter: one polypeptide having 65 amino acids including translation starting methionine, N-terminal 60 amino acids of human IL-2 and 4 amino acids coded for by an intercistronic sequence, and the other having 152 amino acids including translation starting methionine and human M-CSF with 151 amino acids from 35th amino acid (Val) to 185th amino acid (Thr) shown in Fig. 5.

In this two-cistron expression system, translation of the second cistron is initiated by the attachment of ribosome to the second SD sequence positioned in the intercistronic sequence.

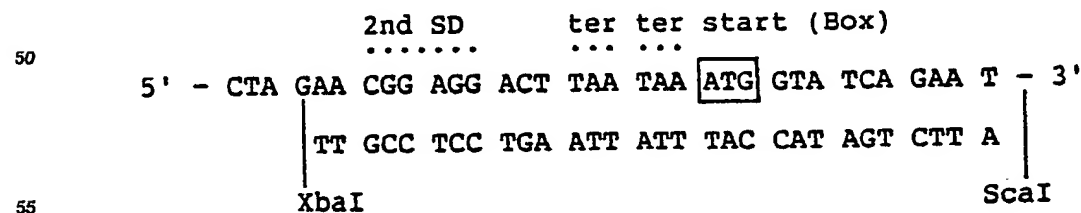
Fig. 15 schematically shows the structure of the above two-cistron expression system.

(2) Preparation of M-CSF expression plasmid ptrpIL-2X*M*CSF102

The desired plasmid ptrpIL-2X*M*CSF102 different from the above plasmid only in intercistronic sequence was prepared in the same manner as in procedure (1) except that the linker (II) given below was used.

Fig. 16 shows the structure of the plasmid.

Synthetic linker (II):



containing M-CSF.

The cultures of *E. coli* transformed with the other plasmids of procedures (2) to (4) were similarly analyzed in the same manner as above to confirm that they produced the translation product (M-CSF) of the second cistron.

5

(6) Separation and purification of M-CSF

10 1) Preparation of M-CSF fraction from *E. coli*

To 1.5 g (wet weight) of *E. coli* harboring the plasmid ptpIL-2X*M*CSF10 obtained by procedure (1) was added 50 ml of 50 mM Tris HCl buffer (pH 7.0) containing 0.5 M sucrose, and the mixture was thoroughly stirred. Next, 6 ml of lysozyme (2 mg/ml) and then 4 ml of 0.14M EDTA were added to the mixture, followed by stirring at 4°C for 15 minutes. The mixture was thereafter centrifuged at 10000 r.p.m. for 20 minutes.

The supernatant was discarded, and the precipitate was washed with the same buffer as above (50 mM Tris HCl containing 0.5 M sucrose, pH 7.0) and similarly centrifuged at 10000 r.p.m. for 20 minutes to obtain spheroplasts as a precipitate.

20 Subsequently, 50 ml of 50 mM Tris HCl (pH 7.0) was added to the precipitate to obtain a suspension, which was sonicated at 20 kHz for 10 minutes, followed by centrifugation at 10000 r.p.m. for 20 minutes, then by washing with the same buffer (50 mM Tris HCl, pH 7.0) and thereafter by centrifugation again under the same condition as above, whereby an M-CSF fraction was obtained as precipitate.

25

2) Refolding of M-CSF from M-CSF fraction

To the M-CSF fraction obtained by the above step 1) was added 100 ml of 50 mM Tris HCl (pH 7.0) containing 7.0 M guanidine hydrochloride, and the mixture was stirred at 4°C for 1 hour to obtain a solution. The solution was slowly added dropwise into a beaker containing 300 ml of 10 mM Tris HCl (pH 8.5) with stirring. The mixture was thereafter thoroughly dialyzed against 10 mM Tris HCl (pH 8.5) at 4°C. The dialyzate was centrifuged at 10000 r.p.m. for 20 minutes to remove the precipitate and obtain a supernatant.

Refolded M-CSF was present in the supernatant.

35

3) Purification of M-CSF

The M-CSF obtained by the step 2) was purified in the same manner as in Example 2, procedure (18) as will be described below.

40

3-1) Gel filtration HPLC

The supernatant obtained by the step 2) above was concentrated by a ultrafiltration device (product of Amicon, membrane: YM-10, product of Amicon), the concentrate was passed through 0.45-μm Millipore Filter and subjected to gel filtration HPLC under the following conditions.

Column : TSKgel G3000SW (60 cm x 21.5 mm I.D., product of TOSOH CORPORATION)

Eluent : 50 mM sodium phosphate buffer (pH 6.8) containing 0.3 M NaCl

Flow rate : 3.0 ml/min

50 Fraction volume: 6 ml/tube/2 min

From the above procedure, the molecular weight of M-CSF was estimated to be 32000 in view of the eluted positions of gel filtration HPLC standard proteins (products of ORIENTAL YEAST CO., LTD.), i.e., glutamate dehydrogenase (290000 in molecular weight), lactate dehydrogenase (142000 in molecular weight), enolase (67000 in molecular weight) and adenylate kinase (32000 in molecular weight).

55 The active fraction was collected and buffer-exchanged to 40 mM sodium borate (pH 8.0) by ultra-filtration.

3-2) TSKgel DEAE-5PW ion exchange HPLC

The active fraction obtained by the step 3-1) was subjected to TSKgel DEAE-5PW ion exchange HPLC under the following conditions.

- 5 Column : TSKgel DEAE-5PW (7.5 mm I.D. x 75 mm, product of TOSOH CORPORATION)
 Eluent A : 40 mM sodium borate buffer (pH 8.0) containing 5% methanol
 Eluent B : 40 mM sodium borate buffer (pH 8.0) containing 1.0 M NaCl and 5% methanol
 Flow rate : 1.0 ml/min
 Fraction volume: 1.0 ml/tube/min
 10 Gradient programn:

	Time (min)	%B
15	0	0
	7	0
	42	30
	47	100
	52	100
20	57	0
	62	0

Fig. 21 shows the result of the chromatography (elution profile). In the figure, plotted as ordinate are the absorbance (A_{280}) of protein at 280 nm and the NaCl concentration (M) vs. the fraction No. as abscissa. The double arrow (\leftrightarrow) shown indicates M-CSF activity.

The peak for fractions No.42 and No.43 (NaCl concentration 0.23 to 0.25 M) corresponds to M-CSF activity.

These fractions were collected to obtain M-CSF from E. coli.

30 3-3) N-terminal amino acid sequence of M-CSF

In the same manner as in Example 2, procedure (18), 6), the N-terminal amino acid sequence of the M-CSF obtained above was determined.

35 Consequently, the following sequence was determined.
 Met-Val-Ser-Glu-Tyr-

40 4) SDS-PAGE of M-CSF

In the same manner as in Example 2, procedure (18), 7) except for using molecular weight marker from Pharmacia Fine Chemicals (electrophoresis calibration kit), the molecular weight of M-CSF prepared by step 3-2) was determined by SDS-PAGE.

45 From the result of SDS-PAGE, the molecular weight of M-CSF was estimated to be about 29500 under nonreducing condition (2-ME⁻) and about 17400 under reducing condition (2-ME⁺). The CSF was electrophoresed as a single band at these positions.

50 5) Refolding of M-CSF

To 7.5 g (wet weight) of E. coli harboring the plasmid ptpIL-2X*M*CSF101 obtained by the procedure (1) was added 50 mM Tris HCl buffer (pH 7.0) containing 0.5 M sucrose to obtain 100 ml of suspension, which was then thoroughly stirred. Next, 6 ml of lysozyme (24 mg/ml) and then 4 ml of 0.14 M EDTA were added to the suspension, followed by stirring at 4°C for 15 minutes and then by centrifugation at 10000 r.p.m. for 20 minutes.

The supernatant was discarded, and the precipitate was washed with the same buffer as above (50 mM Tris HCl containing 0.5 M sucrose, pH 7.0) and similarly centrifuged at 10000 r.p.m. for 20 minutes to obtain spheroplasts as a precipitate.

Subsequently, 100 ml of 50 mM Tris HCl (pH 7.0) was added to the precipitate to obtain a suspension, which was sonicated (200 kHz, 2 minutes, 200 W) and then centrifuged at 10000 r.p.m. for 20 minutes. The resulting precipitate was thoroughly washed with 50 mM Tris HCl (pH 7.5) containing 2% Triton X-100 and then centrifuged again under the same condition to obtain an M-CSF fraction as precipitate.

- 5 To the M-CSF fraction obtained was added 20 ml of 50 mM Tris HCl (pH 7.5) containing 7.0 M guanidine hydrochloride and 25 mM 2-mercaptoethanol, and the mixture was stirred at room temperature for 4 hours to reduce, denature and solubilize the protein. The solution was slowly placed dropwise into a beaker containing 2000 ml of 40 mM Tris HCl (pH 8.5), with 0.5 mM reduced glutathione, 0.1 mM oxidized glutathione and 2 mM urea contained therein, while stirring the solution to obtain a 100-fold diluted solution.
- 10 The solution was thereafter allowed to stand at 4 °C for 1 to 2 days to obtain refolded M-CSF.

6) Isoelectric point of M-CSF

- 15 The isoelectric point of the M-CSF prepared by step 5) was determined by the following method.

A 100 µl quantity of M-CSF was applied to a gel (Ampholine PAG plate, pH 3.5-9.5, product of LKB) and electrophoresed at 1W per centimeter (width) of gel. After the current value became constant, the electrophoresis was further continued for 30 minutes. During electrophoresis, the gel was maintained at 10 °C using a cooler (COOLFLOW CFT-25, product of NESLAB Instruments Inc.).

- 20 Gel was thereafter sliced at a spacing of 5 mm, then placed into 1 ml of 50 mM sodium borate (pH 8.0) and eluted for 24 hours. The CSF activity of the eluate was determined.

The gel was also sliced at a spacing of 1 cm, and then stood in 1 ml of distilled water for 24 hours. The pH of the water was then measured by a pH meter.

Consequently, the isoelectric point of M-CSF was found to be pH 4.8.

25

Claims

1. A biologically active recombinant human M-CSF obtained by expressing a human M-CSF gene
 30 coding for the amino acid sequence of the following formula (1) in its entirety or devoid of a portion thereof.
 Formula (1):

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	1	10
	Met-Thr-Ala-Pro-Gly-Ala-Ala-Gly-Arg-Cys-	20
	Pro-Pro-Thr-Thr-Trp-Leu-Gly-Ser-Leu-Leu-	30
5	Leu-Leu-Val-Cys-Leu-Leu-Ala-Ser-Arg-Ser-	40
	Ile-Thr-Glu-Glu-Val-Ser-Glu-Tyr-Cys-Ser-	50
10	His-Met-Ile-Gly-Ser-Gly-His-Leu-Gln-Ser-	60
	Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-	70
	Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-	80
15	Asp-Gln-Glu-Gln-Leu-Lys-Asp-Pro-Val-Cys-	90
	Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-	100
20	X -Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-	110
	Asp-Asn-Thr-Pro-Asn-Ala-Ile-Ala-Ile-Val-	120
	Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys-	130
25	Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-	140
	Asp-Lys-Ala-Cys-Val-Arg-Thr-Phe-Tyr-Glu-	150
30	Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-	160
	Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-	170
	Asp-Lys-Asp-Trp-Asn-Ile-Phe-Ser-Lys-Asn-	180
35	Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-	190
	Gln-Asp-Val-Val-Thr-Lys-Pro-Asp-Cys-Asn-	

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200
 Cys-Leu-Tyr-Pro-Lys-Ala-Ile-Pro-Ser-Ser-
 210
 Asp-Pro-Ala-Ser-Val-Ser-Pro-His-Gln-Pro-
 5 220
 Leu-Ala-Pro-Ser-Met-Ala-Pro-Val-Ala-Gly-
 230
 Leu-Thr-Trp-Glu-Asp-Ser-Glu-Gly-Thr-Glu-
 240
 10 Gly-Ser-Ser-Leu-Leu-Pro-Gly-Glu-Gln-Pro-
 250
 Leu-His-Thr-Val-Asp-Pro-Gly-Ser-Ala-Lys-
 260
 Gln-Arg-Pro-Pro-Arg-Ser-Thr-Cys-Gln-Ser-
 15 270
 Phe-Glu-Pro-Pro-Glu-Thr-Pro-Val-Val-Lys-
 280
 Asp-Ser-Thr-Ile-Gly-Gly-Ser-Pro-Gln-Pro-
 290
 20 Arg-Pro-Ser-Val-Gly-Ala-Phe-Asn-Pro-Gly-
 300
 Met-Glu-Asp-Ile-Leu-Asp-Ser-Ala-Met-Gly-
 310
 Thr-Asn-Trp-Val-Pro-Glu-Glu-Ala-Ser-Gly-
 25 320
 Glu-Ala-Ser-Glu-Ile-Pro-Val-Pro-Gln-Gly-
 330
 Thr-Glu-Leu-Ser-Pro-Ser-Arg-Pro-Gly-Gly-
 340
 30 Gly-Ser-Met-Gln-Thr-Glu-Pro-Ala-Arg-Pro-
 350
 Ser-Asn-Phe-Leu-Ser-Ala-Ser-Ser-Pro-Leu-
 360
 Pro-Ala-Ser-Ala-Lys-Gly-Gln-Gln-Pro-Ala-
 35 370
 Asp-Val-Thr-Gly-Thr-Ala-Leu-Pro-Arg-Val-
 380
 Gly-Pro-Val-Arg-Pro-Thr-Gly-Gln-Asp-Trp-
 390
 40 Asn-His-Thr-Pro-Gln-Lys-Thr-Asp-His-Pro-
 400
 Ser-Ala-Leu-Leu-Arg-Asp-Pro-Pro-Glu-Pro-
 410
 Gly-Ser-Pro-Arg-Ile-Ser-Ser-Pro-Arg-Pro-
 420
 45 Gln-Gly-Leu-Ser-Asn-Pro-Ser-Thr-Leu-Ser-
 430
 Ala-Gln-Pro-Gln-Leu-Ser-Arg-Ser-His-Ser-
 440
 50 Ser-Gly-Ser-Val-Leu-Pro-Leu-Gly-Glu-Leu-

55

450
 Glu-Gly-Arg-Arg-Ser-Thr-Arg-Asp-Arg-Arg-
 460
 Ser-Pro-Ala-Glu-Pro-Glu-Gly-Gly-Pro-Ala-
 470
 5 Ser-Glu-Gly-Ala-Ala-Arg-Pro-Leu-Pro-Arg-
 480
 Phe-Asn-Ser-Val-Pro-Leu-Thr-Asp-Thr-Gly-
 490
 10 His-Glu-Arg-Gln-Ser-Glu-Gly-Ser-Ser-Ser-
 500
 Pro-Gln-Leu-Gln-Glu-Ser-Val-Phe-His-Leu-
 510
 Leu-Val-Pro-Ser-Val-Ile-Leu-Val-Leu-Leu-
 520
 15 Ala-Val-Gly-Gly-Leu-Leu-Phe-Tyr-Arg-Trp-
 530
 Arg-Arg-Arg-Ser-His-Gln-Glu-Pro-Gln-Arg-
 540
 20 Ala-Asp-Ser-Pro-Leu-Glu-Gln-Pro-Glu-Gly-
 550
 Ser-Pro-Leu-Thr-Gln-Asp-Asp-Arg-Gln-Val-
 554
 Glu-Leu-Pro-Val

25

wherein X is Tyr or Asp.

2. A recombinant human M-CSF as defined in claim 1 wherein the human M-CSF gene is one possessed by the human M-CSF expression plasmids ptrpIL-2X*M*CSF101, ptrpIL-2X*M*CSF104, pcDM*CSF11-dhfr, pcDM*CSF11-185dhfr, pcDM*CSF11-177, pcDM*CSF11-163 and pcDM*CSF.

3. A process for preparing a recombinant human M-CSF comprising cultivating a transformant containing the human M-CSF gene defined in claim 1 and harboring an expression plasmid capable of expressing a biologically active human M-CSF and collecting the human M-CSF produced.

4. A process as defined in claim 3 wherein the transformant is obtained using CHO cell as a host.

5. A process as defined in claim 4 wherein the expression plasmid is selected from among pcDM*CSF11-dhfr, pcDM*CSF11-dhfrRo, pcDM*CSF11-185-dhfr, pRSVS-MCSF11-dhfr and pSV2S-MCSF11-dhfr.

6. A recombinant human M-CSF obtained by the process defined in claim 5.

7. A recombinant human M-CSF as defined in claim 1 which has the following physicochemical properties.

(a) Molecular weight:

About 38000 when determined in the presence of 2-mercaptoethanol and about 76000 when determined in the absence of 2-mercaptoethanol, by SDS-PAGE.

(b) Isoelectric point: pH 4.55.

(c) N-terminal amino acid sequence:

Glu-Glu-Val-Ser-Glu-Tyr.

8. A recombinant human M-CSF as defined in claim 1 which has the following physicochemical properties.

(a) Molecular weight:

About 22000 and about 27000 when determined in the presence of 2-mercaptoethanol and about 42000 and about 46000 when determined in the absence of 2-mercaptoethanol, by SDS-PAGE.

(b) Isoelectric point: pH 3.5 - 4.6.

(c) N-terminal amino acid sequence:

Glu-Glu-Val-Ser-Glu-Tyr.

9. A process as defined in claim 3 wherein the transformant is obtained using COS cell as a host.

- 10 A process as defined in claim 9 wherein the expression plasmid is selected from among pcDM*CSF, pcDM*CSF-185, pcDM*CSF-177, pcDM*CSF11, pcDM*CSF11-185, pcDM*CSF11-177 and pcDM*CSF11-163.
11. A recombinant human M-CSF obtained by the process defined in claim 10.
- 5 12. A process as defined in claim 3 wherein the transformant is obtained using *E. coli* as a host.
13. A process as defined in claim 12 wherein the human M-CSF is expressed by a two-cistron system.
14. A process as defined in claim 13 wherein the expression plasmid is selected from among ptrpIL-2X*M*CSF101, ptrpIL-2X*M*CSF102, ptrpIL-2X*M*CSF103, ptrpIL-2X*M*CSF104, ptrpIL-2X*M*CSF105 and ptrpIL-2X*M*CSF106.
- 10 15. A process as defined in claim 12 wherein the human M-CSF is expressed by secretory expression utilizing a signal peptide.
16. A process as defined in claim 15 wherein the expression plasmid is pIN-III(1pp^P-5)-OmpA-MCSF11-NV151 or pIN-III(1pp^P-5)-OmpA-MCSF11-NV143.
17. A recombinant human M-CSF obtained by the process of claim 16.
- 15 18. A recombinant human M-CSF as defined in claim 1 which has the following physicochemical properties.
- (a) Molecular weight:
About 17000 when determined in the presence of 2-mercaptoethanol and about 30000 when determined in the absence of 2-mercaptoethanol, by SDS-PAGE.
- 20 (b) Isoelectric point: pH 4.8.
- (c) N-terminal amino acid sequence:
(Met)-Val-Ser-Glu-Tyr-
wherein (Met) is present or absent.
- 25 19. An expression plasmid containing the human M-CSF gene defined in claim 1 and capable of expressing a biologically active human M-CSF.
20. An expression plasmid as defined in claim 19 which is ptrpIL-2X*M*CSF101, ptrpIL-2X*M*CSF102, ptrpIL-2X*M*CSF103, ptrpIL-2X*M*CSF104, ptrpIL-2X*M*CSF105, ptrpIL-2X*M*CSF106, pcDM*CSF11-dhfr, pcDM*CSF11-dhfrRO, pcDM*CSF11-185-dhfr, pRSVS-MCSF11-dhfr, pSV2S-MCSF11-dhfr, pcDM*CSF11, pcDM*CSF11-185, pcDM*CSF11-177, pcDM*CSF11-163, pcDM*CSF, pcDM*CSF-185, pcDM*CSF-177, pIN-III(1pp^P-5)-OmpA-MCSF11-NV151 or pIN-III(1pp^P-5)-OmpA-MCSF11-NV143.
- 30 21. A transformant containing the human M-CSF gene defined in claim 1 and harboring an expression plasmid capable of expressing a biologically active human M-CSF.
22. The use of human M-CSF according to claims 1, 2, 7, 8 and 18 for the preparation of a pharmaceutical useful in treatment of leukopenia and against infectious diseases.
- 35
- 40
- 45
- 50
- 55

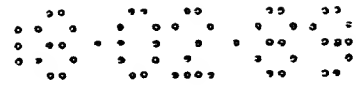
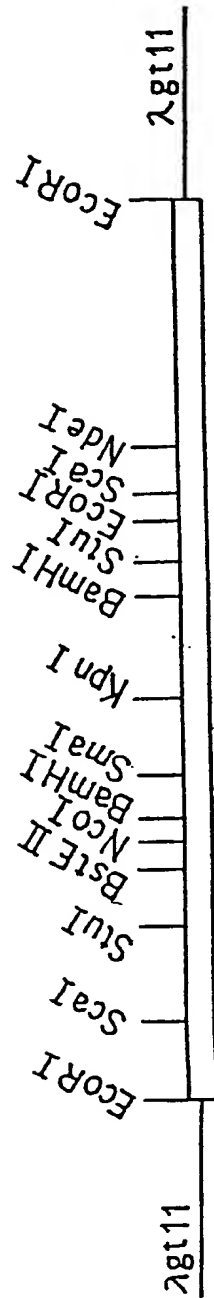


FIG. 1



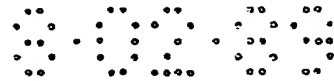


FIG. 2-1

5' AGCCGCTCTC CGCATCCCAG GACAGCGGTG CGGCCCTCGG CCGGGGCGCC
 TCGGCGAGAG GCGTAGGGTC CTGTGCCAC GCCGGGAGCC GGCCCCGCGG

 CACTCCGCAG CACCCAGCGA GCGAGCGAGC GAGCGAGGGC GGCCGACGCG
 GTGAGGCGTC GTGGGTCGCT CGCTCGCTCG CTCGCTCCCC CCGGCTGCGC

 CCCGGCCGGG ACCCAGCTGC CCGTATGACC GCGCCGGGCG CCGCCGGGCG
 GGGCCGGCCC TGGGTCGACG GGCACTACTGG CGCGGCCCGC GGCGGCCCGC

 CTGCCCTCCC ACGACATGGC TGGGCTCCCT GCTGTTGTTG GTCTGTCTCC
 GACGGGAGGG TGCTGTACCG ACCCGAGGGA CGACAACAAC CAGACAGAGG

 TGGCGAGCAG GAGTATCACC GAGGAGGTGT CGGAGTACTG TAGCCACATG
 ACCGCTCGTC CTCATAGTGG CTCTCCACA GCCTCATGAC ATCGGTGTAC

 ATTGGGAGTG GACACCTGCA GTCTCTGCAG CGGCTGATTG ACAGTCAGAT
 TAACCCTCAC CTGTGGACGT CAGAGACGTC GCCGACTAAC TGTCAGTCTA

 GGAGACCTCG TGCCAAATTA CATTTGAGTT TGTAGACCAG GAACAGTTGA
 CCTCTGGAAC ACGGTTTAAT GTAAACTCAA ACATCTGGTC CTTGTCAACT

 AAGATCCAGT GTGCTACCTT AAGAAGGCAT TTCTCCTGGT ACAATACATA
 TTCTAGGTCA CACGATGGAA TTCTTCCGTA AAGAGGACCA TGTTATGTAT

 ATGGAGGACA CCATGCGCTT CAGAGATAAC ACCCCCAATG CCATCGCCAT
 TACCTCCTGT GGTACGCGAA GTCTCTATTG TGGGGGTTAC GGTAGCGGTA

 TGTGCAGCTG CAGGAACTCT CTTTGAGGCT GAAGAGCTGC TTCACCAAGG
 ACACGTCGAC GTCCTTGAGA GAAACTCCGA CTTCTCGACG AAGTGGTTCC

 ATTATGAAGA GCATGACAAG GCCTGCGTCC GAACTTTCTA TGAGACACCT
 TAATACTTCT CGTACTGTTC CGGACGCAGG CTTGAAAGAT ACTCTGTGGA

 CTCCAGTTGC TGGAGAAGGT CAAGAATGTC TTTAATGAAA CAAAGAATCT
 GAGGTCAACG ACCTCTTCCA GTTCTTACAG AAATTACTTT GTTCTTAGA

 CCTTGACAAG GACTGGAATA TTTTCAGCAA GAACTGCAAC AACAGCTTTG
 GGAAGTGTTC CTGACCTTAT AAAAGTCGTT CTTGACGTTG TTGTCGAAAC

 CTGAATGCTC CAGCCAAGAT GTGGTGACCA AGCCTGATTG CAACTGCCTG
 GACTTACGAG GTCGGTTCTA CACCACTGGT TCGGACTAAC GTTGACGGAC

 TACCCCAAAG CCATCCCTAG CAGTGACCCG GCCTCTGTCT CCCCTCATCA
 ATGGGGTTTC GGTAGGGATC GTCAGTGGC CGGAGACAGA GGGGAGTAGT

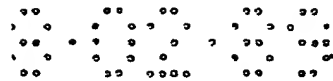


FIG. 2-2

GCCCCTCGCC	CCCTCCATGG	CCCCTGTGGC	TGGCTTGACC	TGGGAGGACT
CGGGGAGCGG	GGGAGGTACC	GGGGACACCG	ACCGAACTGG	ACCCTCCTGA
CTGAGGGAAC	TGAGGGCAGC	TCCCTCTTGC	CTGGTGAGCA	GCCCCTGAC
GACTCCCTTG	ACTCCCGTCG	AGGGAGAACG	GACCACTCGT	CGGGGACGTG
ACAGTGGATC	CAGGCAGTGC	CAAGCAGCGG	CCACCCAGGA	GCACCTGCCA
TGTCACCTAG	GTCCGTCACG	GTTCTGTCGC	GGTGGGTCCT	CGTGGACGGT
GAGCTTTGAG	CCGCCAGAGA	CCCCAGTTGT	CAAGGACAGC	ACCATCGGTG
CTCGAAACTC	GGCGGTCTCT	GGGGTCAACA	GTTCTGTCTG	TGGTAGCCAC
GCTCACCACA	GCCTCGCCCC	TCTGTGCGGG	CCTTCAACCC	CGGGATGGAG
CGAGTGGTGT	CGGAGCGGGG	AGACAGCCCC	GGAAGTTGGG	GCCCTACCTC
GATATTCTTG	ACTCTGCAAT	GGGCACTAAT	TGGGTCCCAG	AAGAAGCCTC
CTATAAGAAC	TGAGACGTTA	CCCGTGATTA	ACCCAGGGTC	TTCTTCGGAG
TGGAGAGGCC	AGTGAGATTC	CCGTACCCCA	AGGGACAGAG	CTTTCCCCCT
ACCTCTCCGG	TCACTCTAAG	GGCATGGGGT	TCCCTGTCTC	GAAAGGGGGA
CCAGGCCAGG	AGGGGGCAGC	ATGCAGACAG	AGCCCGCCAG	ACCCAGCAAC
GGTCCGGTCC	TCCCCCGTCG	TACGTCTGTC	TCGGGCGGTC	TGGGTGCTTG
TTCCTCTCAG	CATCTTCTCC	ACTCCCTGCA	TCAGCAAAGG	GCCAACAGCC
AAGGAGAGTC	GTAGAAGAGG	TGAGGGACGT	AGTCGTTTCC	CGGTTGTCTG
GGCAGATGTA	ACTGGTACCG	CCTTGCCCAG	GGTGGGCCCC	TGAGCATGGC
CCGTCTACAT	TGACCATGGC	GGAACGGGTC	CCACCCGGGC	ACTCGTACCG
CAGGACTGGA	ATCACACCCC	CCAGAAGAGA	CACCATCCAT	CTGCCCTGCT
GTCCTGACCT	TAGTGTGGGG	GGTCTTCTCT	GTGGTAGGTA	GACGGGACGA
CAGAGACCCC	CCGGAGCCAG	GCTCTCCCAG	GATCTCATCA	CCGCGCCCCC
GTCTCTGGGG	GGCCTCGGTC	CGAGAGGGTC	CTAGAGTAGT	GGCGCGGGGG
AGGGCCTCAG	CAACCCCTCC	ACCCTCTGCT	GCTCAGCCAC	AGCTTTCCAG
TCCCGGAGTC	GTTGGGGAGG	TGGGAGACGA	CGAGTCGGTG	TCGAAAGGTC
AAGCCACTCC	TCGGGCGTGC	TGCCCTTGGG	GAGCTGGAGG	GCAGGAGGAG
TTCGGTGAGG	AGCCCGCACG	ACGGGAACCC	CTCGACCTCC	CGTCCTCCTC
CACCAGGGAT	CGGAGGAGCC	CCGCAGAGCC	AGAAGGAGGA	CCAGCAAGTG
GTGGTCCCTA	GCCTCCTCGG	GGCGTCTCGG	TCTTCCTCCT	GGTCGTTTAC



FIG. 2-3

AAGGCAGCCA	GCCCCTGCCC	CGTTTAACT	CCGTTCCCTT	GACTGACACA
TTCCGTCGGT	CGGGGACGGG	GCAAAATTGA	GGCAAGGAAA	CTGACTGTGT
GGCCATGAGA	GGCAGTCCGA	GGGATCCTCC	AGCCCGCAGC	TCCAGGAGTC
CCGGTACTCT	CCGTCAGGCT	CCCTAGGAGG	TCGGGCGTCG	AGGTCCTCAG
TGTCTTCCAC	CTGCTGGTGC	CCAGTGTCT	CCTGGTCTTG	CTGGCCGTCTG
ACAGAAGGTG	GACGACCACG	GGTCACAGTA	GGACCAGAAC	GACCGGCAGC
GAGGCCTCTT	GTTCTACAGG	TGGAGGCGGC	GGAGCCATCA	AGAGCCTCAG
CTCCGGAGAA	CAAGATGTCC	ACCTCCGCCG	CCTCGGTAGT	TCTCGGAGTC
AGAGCGGATT	CTCCCTTGGA	GCAACCAGAG	GGCAGCCCCC	TCACTCAGGA
TCTCGCCTAA	GAGGGAACCT	CGTTGGTCTC	CCGTCGGGGG	AGTGAGTCCT
TGACAGACAG	GTGGAAGTGC	CAGTGTAGAG	GGAATTCTAA	GACCCCTCAC
ACTGTCTGTC	CACCTTGACG	GTCACATCTC	CCTTAAGATT	CTGGGGAGTG
CATCCTGGAC	ACTCTCGTTT	GTCAATGTCC	CTCTGAAAAT	GTGACGCCCCA
GTAGGACCTG	TGAGAGCAAA	CAGTTACAGG	GAGACTTTTA	CACTGCGGGT
GCCCCGGACA	CAGTACTCCA	GATGTTGTCT	GACCAGCTCA	GAGAGAGTAC
CGGGGCCTGT	GTCATGAGGT	CTACAACAGA	CTGGTCGAGT	CTCTCTCATG
AGTGGGACTG	TTACCTTCCT	TGATATGGAC	AGTATTCTTC	TATTTGTGCA
TCACCCTGAC	AATGGAAGGA	ACTATACCTG	TCATAAGAAG	ATAAACACGT
GATTAAGATT	GCATTAGTTT	TTTTCTTAAC	AACTGCATCA	TACTGTTGTC
CTAATTCTAA	CGTAATCAAA	AAAAGAATTG	TTGACGTAGT	ATGACAACAG
ATATGTTGAG	CCTGTGGTCT	ATAAAACCCC	TAGTTCATT	TCCCATAAAC
TATACAACCTC	GGACACCAGA	TATTTTGGGG	ATCAAGGTAA	AGGGTATTTG
TTCTGTCAAG	CCAGACCATC	TCTACCCTGT	ACTTGGACAA	CTTAACCTTTT
AAGACAGTTC	GGTCTGGTAG	AGATGGGACA	TGAACCTGTT	GAATTGAAAA
TTAACCAAAG	TGCAGTTTAT	GTTACCTTTT	GTAAAGCCA	CCTTGTGGTT
AATTGGTTTC	ACGTCAAATA	CAAGTGGA	CAATTTCTGGT	GGAACACCAA
TCTGCCCATC	ACCTGAACCT	ACTGAAGTTG	TGTGAAATCC	TAATTCTGTC
AGACGGGTAG	TGGACTTGGA	TGACTTCAAC	ACACTTTAGG	ATTAAGACAG
ATCTCCGTAG	CCCTCCAGT	TGTGCCTCCT	GCACATTGAT	GAGTGCCTGC
TAGAGGCATC	GGGAGGGTCA	ACACGGAGGA	CGTGTAATA	CTCACGGACG



FIG. 2-4

TGTTGTCTTT GCCCATGTTG TTGATGTAGC TGTGACCCTA TTGTTCCCTCA
ACAACAGAAA CGGGTACAAC AACTACATCG AACTGGGAT AACAAGGAGT

CCCCTGCCCC CCGCCAACCC CAGCTGGCCC ACCTCTTCCC CCTCCCACCC
GGGGACGGGG GGCGGTTGGG GTCGACCGGG TGGAGAAGGG GGAGGGTGGG

AAGCCCACAG CCAGCCCATC AGGAAGCCTT CCTGGCTTCT CCACAACCTT
TTCGGGTGTC GGTCTGGGTAG TCCTTCGGAA GGACCGAAGA GGTGTTGGAA

CTGACTGTCT TTTCAGTCAT GCCCCCTGCT CTTTGTATT TGGCTAATAG
GACTGACAGA AAAGTCAGTA CGGGGGACGA GAAAACATAA ACCGATTATC

TATATCAATT TGCACCTAAA AAAAAAAAAA AAAAAAAAA 3'
ATATAGTTAA ACGTGAATTT TTTTTTTTTT TTTTTTTT

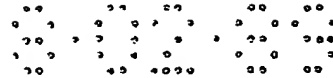


FIG. 3-1

ATG.ACC.GCG.CCG.GGC.GCC.GCC.GGG.CGC.TGC.CCT.CCC.ACG.ACA.TGG.
 Met-Thr-Ala-Pro-Gly-Ala-Ala-Gly-Arg-Cys-Pro-Pro-Thr-Thr-Trp-
 CTG.GGC.TCC.CTG.CTG.TTG.TTG.GTC.TGT.CTC.CTG.GCG.AGC.AGG.AGT.
 Leu-Gly-Ser-Leu-Leu-Leu-Leu-Val-Cys-Leu-Leu-Ala-Ser-Arg-Ser-
 ATC.ACC.GAG.GAG.GTG.TCG.GAG.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
 Ile-Thr-Glu-Glu-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
 GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
 Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-
 ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
 Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-
 AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
 Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-
 TAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
 Tyr-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-
 GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
 Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys
 AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
 Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-
 CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
 Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-
 AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
 Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-
 ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
 Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-
 CAA.GAT.GTG.GTG.ACC.AAG.CCT.GAT.TGC.AAC.TGC.CTG.TAC.CCC.AAA.
 Gln-Asp-Val-Val-Thr-Lys-Pro-Asp-Cys-Asn-Cys-Leu-Tyr-Pro-Lys-
 GCC.ATC.CCT.AGC.AGT.GAC.CCG.GCC.TCT.GTC.TCC.CCT.CAT.CAG.CCC.
 Ala-Ile-Pro-Ser-Ser-Asp-Pro-Ala-Ser-Val-Ser-Pro-His-Gln-Pro-
 CTC.GCC.CCC.TCC.ATG.GCC.CCT.GTG.GCT.GGC.TTG.ACC.TGG.GAG.GAC.
 Leu-Ala-Pro-Ser-Met-Ala-Pro-Val-Ala-Gly-Leu-Thr-Trp-Glu-Asp-



FIG. 3-2

TCT.GAG.UGA.ACT.GAG.GGC.AGC.TCC.CTC.TTG.CCT.GGT.GAG.CAG.CCC.
Ser-Glu-Gly-Thr-Glu-Gly-Ser-Ser-Leu-Leu-Pro-Gly-Glu-Gln-Pro-

CTG.CAC.ACA.GTG.GAT.CCA.GGC.AGT.GCC.AAG.CAG.CGG.CCA.CCC.AGG.
Leu-His-Thr-Val-Asp-Pro-Gly-Ser-Ala-Lys-Gln-Arg-Pro-Pro-Arg-

AGC.ACC.TGC.CAG.AGC.TTT.GAG.CCG.CCA.GAG.ACC.CCA.GTT.GTC.AAG.
Ser-Thr-Cys-Gln-Ser-Phe-Glu-Pro-Pro-Glu-Thr-Pro-Val-Val-Lys-

GAC.AGC.ACC.ATC.GGT.GGC.TCA.CCA.CAG.CCT.CGC.CCC.TCT.GTC.GGG.
Asp-Ser-Thr-Ile-Gly-Gly-Ser-Pro-Gln-Pro-Arg-Pro-Ser-Val-Gly-

GCC.TTC.AAC.CCC.GGG.ATG.GAG.GAT.ATT.CTT.GAC.TCT.GCA.ATG.GGC.
Ala-Phe-Asn-Pro-Gly-Met-Glu-Asp-Ile-Leu-Asp-Ser-Ala-Met-Gly-

ACT.AAT.TGG.GTC.CCA.GAA.GAA.GCC.TCT.GGA.GAG.GCC.AGT.GAG.ATT.
Thr-Asn-Trp-Val-Pro-Glu-Glu-Ala-Ser-Gly-Glu-Ala-Ser-Glu-Ile-

CCC.GTA.CCC.CAA.GGG.ACA.GAG.CTT.TCC.CCC.TCC.AGG.CCA.GGA.GGG.
Pro-Val-Pro-Gln-Gly-Thr-Glu-Leu-Ser-Pro-Ser-Arg-Pro-Gly-Gly-

GGC.AGC.ATG.CAG.ACA.GAG.CCC.GCC.AGA.CCC.AGC.AAC.TTC.CTC.TCA.
Gly-Ser-Met-Gln-Thr-Glu-Pro-Ala-Arg-Pro-Ser-Asn-Phe-Leu-Ser-

GCA.TCT.TCT.CCA.CTC.CCT.GCA.TCA.GCA.AAG.GGC.CAA.CAG.CCG.GCA.
Ala-Ser-Ser-Pro-Leu-Pro-Ala-Ser-Ala-Lys-Gly-Gln-Gln-Pro-Ala-

GAT.GTA.ACT.GGT.ACC.GCC.TTG.CCC.AGG.GTG.GGC.CCG.TGA.
Asp-Val-Thr-Gly-Thr-Ala-Leu-Pro-Arg-Val-Gly-Pro-***-

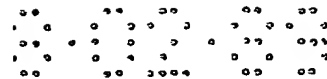


FIG. 4-1

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 TCGGCGAGAG GCGTAGGGTC CTGTCGCCAC GCCGGGAGCC GCGCCCGCGG

 CACTCCGCAG CACCCAGCGA GCGAGCGAGC GAGCGAGGGC GGCCGACGCG
 GTGAGGCGTC GTGGGTCGCT CGCTCGCTCG CTCGCTCCCG CCGGCTGCGC

 CCCGGCCGGG ACCCAGCTGC CCGTATGACC GCGCCGGGCG CCGCCGGGCG
 GGGCCGGCCC TGGGTCGACG GGCATACTGG CGCGGCCCGC GGCGGCCCGC

 CTGCCCTCCC ACGACATGGC TGGGCTCCCT GCTGTTGTTG GTCTGTCTCC
 GACGGGAGGG TGCTGTACCG ACCCGAGGGA CGACAACAAC CAGACAGAGG

 TGGCGAGCAG GAGTATCACC GAGGAGGTGT CGGAGTACTG TAGCCACATG
 ACCGCTCGTC CTCATAGTGG CTCCTCCACA GCCTCATGAC ATCGGTGTAC

 ATTGGGAGTG GACACCTGCA GTCTCTGCAG CGGCTGATTG ACAGTCAGAT
 TAACCCTCAC CTGTGGACGT CAGAGACGTC GCCGACTAAC TGTCAGTCTA

 GGAGACCTCG TGCCAAATTA CATTTGAGTT TGTAGACCAG GAACAGTTGA
 CCTCTGGAGC ACGGTTTAAT GTAAACTCAA ACATCTGGTC CTTGTCAACT

 AAGATCCAGT GTGCTACCTT AAGAAGGCAT TTCTCCTGGT ACAAGACATA
 TTCTAGGTCA CACGATGGAA TTCTTCCGTA AAGAGGACCA TGTTCTGTAT

 ATGGAGGACA CCATGCGCTT CAGAGATAAC ACCCCCAATG CCATCGCCAT
 TACCTCCTGT GGTACGCGAA GTCTCTATTG TGGGGGTTAC GGTAGCGGTA

 TGTGCAGCTG CAGGAActCT CTTTGAGGCT GAAGAGCTGC TTCACCAAGG
 ACACGTCGAC GTCCTTGAGA GAAACTCCGA CTTCTCGACG AAGTGGTTCC

 ATTATGAAGA GCATGACAAG GCCTGCGTCC GAACTTTCTA TGAGACACCT
 TAATACTTCT CGTACTGTTC CGGACGCAGG CTTGAAAGAT ACTCTGTGGA

 CTCCAGTTGC TGGAGAAGGT CAAGAATGTC TTTAATGAAA CAAAGAATCT
 GAGGTCAACG ACCTCTTCCA GTTCTTACAG AAATTACTTT GTTTCTTAGA

 CCTTGACAAG GACTGGAATA TTTTCAGCAA GAACTGCAAC AACAGCTTTG
 GGAAGTGTTC CTGACCTTAT AAAAGTCGTT CTTGACGTTG TTGTCGAAAC

 CTGAATGCTC CAGCCAAGAT GTGGTGACCA AGCCTGATTG CAACTGCCTG
 GACTTACGAG GTCGGTCTA CACCACTGGT TCGGACTAAC GTTGACGGAC

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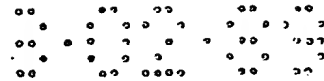


FIG. 4-2

GCCCCCTCGCC	CCCTCCATGG	CCCCTGTGGC	TGGCTTGACC	TGGGAGGACT
CGGGGAGCGG	GGGAGGTACC	GGGGACACCG	ACCGAACTGG	ACCCTCCTGA
CTGAGGGAAC	TGAGGGCAGC	TCCCTCTTGC	CTGGTGAGCA	GCCCCCTGCAC
GACTCCCTTG	ACTCCCGTCG	AGGGAGAACG	GACCACTCGT	CGGGGACGTG
ACAGTGGATC	CAGGCAGTGC	CAAGCAGCGG	CCACCCAGGA	GCACCTGCCA
TGTCACCTAG	GTCCGTCACG	GTTCGTCGCC	GGTGGGTCTT	CGTGGACGGT
GAGCTTTGAG	CCGCCAGAGA	CCCCAGTTGT	CAAGGACAGC	ACCATCGGTG
CTCGAAACTC	GGCGGTCTCT	GGGGTCAACA	GTTCCTGTCT	TGGTAGCCAC
GCTCACCACA	GCCTCGCCCC	TCTGTGCGGG	CCTTCAACCC	CGGGATGGAG
CGAGTGGTGT	CGGAGCGGGG	AGACAGCCCC	GGAAGTTGGG	GCCCTACCTC
GATATTCTTG	ACTCTGCAAT	GGGCACTAAT	TGGGTCCCAG	AAGAAGCCTC
CTATAAGAAC	TGAGACGTTA	CCCCTGATTA	ACCCAGGGTC	TTCTTCGGAG
TGGAGAGGCC	AGTGAGATTC	CCGTACCCCA	AGGGACAGAG	CTTTCCCCCT
ACCTCTCCGG	TCACTCTAAG	GGCATGGGGT	TCCCTGTCTC	GAAAGGGGGA
CCAGGCCAGG	AGGGGGCAGC	ATGCAGACAG	AGCCCGCCAG	ACCCAGCAAC
GGTCCGGTCC	TCCCCCGTCG	TACGTCTGTC	TCGGGCGGTC	TGGGTCTGTTG
TTCTCTCTCAG	CATCTTCTCC	ACTCCCTGCA	TCAGCAAAGG	GCCAACAGCC
AAGGAGAGTC	GTAGAAAGAG	TGAGGGACGT	AGTCGTTTCC	CGGTGTCTCG
GGCAGATGTA	ACTGGTACCG	CCTTGCCCAG	GGTGGGCCCCG	GTGAGGCCCA
CCGTCTACAT	TGACCATGGC	GGAACGGGTC	CCACCCGGGC	CACTCCGGGT
CTGGCCAGGA	CTGGAATCAC	ACCCCCCAGA	AGACAGACCA	TCCATCTGCC
GACCGGTCCCT	GACCTTAGTG	TGGGGGGTCT	TCTGTCTGGT	AGGTAGACGG
CTGCTCAGAG	ACCCCCCGGA	GCCAGGCTCT	CCCAGGATCT	CATCACCGCG
GACGAGTCTC	TGGGGGGCCT	CGGTCCGAGA	GGGTCCTAGA	GTAGTGGCGC
CCCCCAGGGC	CTCAGCAACC	CCTCCACCCT	CTCTGCTCAG	CCACAGCTTT
GGGGGTCCCCG	GAGTCGTTGG	GGAGGTGGGA	GAGACGAGTC	GGTGTGCGAA
CCAGAAGCCA	CTCCTCGGGC	AGCGTGCTGC	CCCTTGGGGA	GCTGGAGGGC
GGTCTTCCGT	GAGGAGCCCCG	TCGCACGACG	GGGAACCCCT	CGACCTCCCCG
AGGAGGAGCA	CCAGGGATCG	GAGGAGCCCC	GCAGAGCCAG	AAGGAGGACC
TCCTCCTCGT	GGTCCCTAGC	CTCCTCGGGG	CGTCTCGGTC	TTCTCCTG

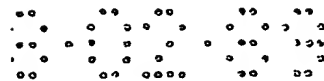


FIG. 4-3

AGCAAGTGAA	GGGGCAGCCA	GGCCCCTGCC	CCGTTTAAAC	TCCGTTTCCTT
TCGTTCACTT	CCCCGTCGGT	CCGGGGACGG	GGCAAAATTG	AGGCAAGGAA
TGACTGACAC	AGGCCATGAG	AGGCAGTCCG	AGGGATCCTC	CAGCCCGCAG
ACTGACTGTG	TCCGGTACTC	TCCGTCAGGC	TCCCTAGGAG	GTCGGGGCGTC
CTCCAGGAGT	CTGTCTTCCA	CCTGCTGGTG	CCCAGTGTC	TCCTGGTCTT
GAGGTCCCTCA	GACAGAAGGT	GGACGACCAC	GGGTCACAGT	AGGACCAGAA
GCTGGCCGTC	GGAGGCCTCT	TGTTCTACAG	GTGGAGGCGG	CGGAGCCATC
CGACCGGCAG	CCTCCGGAGA	ACAAGATGTC	CACCTCCGCC	GCCTCGGTAG
AAGAGCCTCA	GAGAGCGGAT	TCTCCCTTGG	AGCAACCAGA	GGGCAGCCCC
TTCTCGGAGT	CTCTCGCCTA	AGAGGGAACC	TCGTTGGTCT	CCCGTCGGGG
CTCACTCAGG	ATGACAGACA	GGTGGAAGTG	CCAGTGTTAG	GGGAATTCTA
GAGTGAGTCC	TACTGTCTGT	CCACCTTGAC	GGTCACATCT	CCCTTAAGAT
AGACCCCTCA	CCATCCTGGA	CACTCTCGTT	TGTCAATGTC	CCTCTGAAAA
TCTGGGGAGT	GGTAGGACCT	GTGAGACCAA	ACAGTTACAG	GGAGACTTTT
TGTGACGCCC	AGCCCCGGAC	ACAGTACTCC	AGATGTTGTC	TGACCAGCTC
ACACTGCGGG	TCGGGGCCTG	TGTCATGAGG	TCTACAACAG	ACTGGTCGAG
AGAGAGAGTA	CAGTGGGACT	GTTACCTTCC	TTGATATGGA	CAGTATTCTT
TCTCTCTCAT	GTCACCCTGA	CAATGGAAGG	AACTATACCT	GTCATAAGAA
CTATTTGTGC	AGATTAAGAT	TGCATTAGTT	TTTTTCTTAA	CAACTGCATC
GATAAACACG	TCTAATTCTA	ACGTAATCAA	AAAAAGAATT	GTTGACGTAG
ATACTGTTGT	CATATGTTGA	GCCTGTGGTC	TATAAAACCC	CTAGTTCCAT
TATGACAACA	GTATACAAC	CGGACACCAG	ATATTTTGGG	GATCAAGGTA
TTCCCATAAA	CTTCTGTCAA	GCCAGACCAT	CTCTACCCTG	TACTTGGACA
AAGGGTATTT	GAAGACAGTT	CGGTCTGGTA	GAGATGGGAC	ATGAACCTGT
ACTTAACTTT	TTTAACCAAA	GTGCAGTTTA	TGTTACACCTT	TGTTAAAGCC
TGAATTGAAA	AAATTGGTTT	CACGTCAAAT	ACAAGTGGAA	ACAATTTTCGG
ACCTTGTTGGT	TTCTGCCCCAT	CACCTGAACC	TACTGAAGTT	GTGTGAAATC
TGGAACACCA	AAGACGGGTA	GTGGACTTGG	ATGACTTCAA	CACACTTTAG
CTAATTCTGT	CATCTCCGTA	GCCCTCCAG	TTGTGCCTCC	TGCACATTGA
GATTAAGACA	GTAGAGGCAT	CGGGAGGGTC	AACACGGAGG	ACGTGTAAC

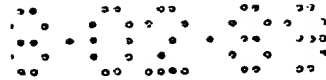


FIG. 4-4

TGAGTGCCTG CTGTTGTCTT TGCCCATGTT GTTGATGTAG CTGTGACCCT
ACTCACGGAC GACAACAGAA ACGGGTACAA CAACTACATC GACACTGGGA

ATTGTTCTTC ACCCCTGCCC CCCGCCAACC CCAGCTGGCC CACCTCTTCC
TAACAAGGAG TGGGGACGGG GGGCGGTTGG GGTCGACCGG GTGGAGAAGG

CCCTCCCACC CAAGCCCACA GCCAGCCCAT CAGGAAGCCT TCCTGGCTTC
GGGAGGGTGG GTTCGGGTGT CGGTCGGGTA GTCCTTCGGA AGGACCGAAG

TCCACAACCT TCTGACTGTC TTTTCAGTCA TGCCCCCTGC TCTTTTGTAT
AGGTGTTGGA AGACTGACAG AAAAGTCAGT ACGGGGGACG AGAAAACATA

TTGGCTAATA GTATATCAAT TTGCACTTAA AAAAAAAAAA AAAAAAAAAA 3'
AACCGATTAT CATATAGTTA AACGTGAATT TTTTTTTTTT TTTTTTTTTT



FIG. 5-1

ATG.ACC.GCG.CCG.GGC.GCC.GCC.GGG.CGC.TGC.CCT.CCC.ACG.ACA.TGG.
 Met-Thr-Ala-Pro-Gly-Ala-Ala-Gly-Arg-Cys-Pro-Pro-Thr-Thr-Trp-
 CTG.GGC.TCC.CTG.CTG.TTG.TTG.GTC.TGT.CTC.CTG.GCG.AGC.AGG.AGT.
 Leu-Gly-Ser-Leu-Leu-Leu-Leu-Val-Cys-Leu-Leu-Ala-Ser-Arg-Ser-
 ATC.ACC.GAG.GAG.GTG.TCG.GAG.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
 Ile-Thr-Glu-Glu-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
 GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
 Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-
 ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
 Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-
 AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
 Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-
 GAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
 Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-
 GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
 Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys
 AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
 Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-
 CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
 Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-
 AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
 Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-
 ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
 Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-
 CAA.GAT.GTG.GTG.ACC.AAG.CCT.GAT.TGC.AAC.TGC.CTG.TAC.CCC.AAA.
 Gln-Asp-Val-Val-Thr-Lys-Pro-Asp-Cys-Asn-Cys-Leu-Tyr-Pro-Lys-
 GCC.ATC.CCT.AGC.AGT.GAC.CCG.GCC.TCT.GTC.TCC.CCT.CAT.CAG.CCC.
 Ala-Ile-Pro-Ser-Ser-Asp-Pro-Ala-Ser-Val-Ser-Pro-His-Gln-Pro-
 CTC.GCC.CCC.TCC.ATG.GCC.CCT.GTG.GCT.GGC.TTG.ACC.TGG.GAG.GAC.
 Leu-Ala-Pro-Ser-Met-Ala-Pro-Val-Ala-Gly-Leu-Thr-Trp-Glu-Asp-

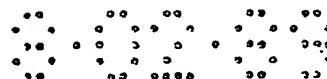


FIG. 5-2

TCT.GAG.GGA.ACT.GAG.GGC.AGC.TCC.CTC.TTG.CCT.GGT.GAG.CAG.CCC.
 Ser-Glu-Gly-Thr-Glu-Gly-Ser-Ser-Leu-Leu-Pro-Gly-Glu-Gln-Pro-
 CTG.CAC.ACA.GTG.GAT.CCA.GGC.AGT.GCC.AAG.CAG.CGG.CCA.CCC.AGG.
 Leu-His-Thr-Val-Asp-Pro-Gly-Ser-Ala-Lys-Gln-Arg-Pro-Pro-Arg-
 AGC.ACC.TGC.CAG.AGC.TTT.GAG.CCG.CCA.GAG.ACC.CCA.GTT.GTC.AAG.
 Ser-Thr-Cys-Gln-Ser-Phe-Glu-Pro-Pro-Glu-Thr-Pro-Val-Val-Lys-
 GAC.AGC.ACC.ATC.GGT.GGC.TCA.CCA.CAG.CCT.CGC.CCC.TCT.GTC.GGG.
 Asp-Ser-Thr-Ile-Gly-Gly-Ser-Pro-Gln-Pro-Arg-Pro-Ser-Val-Gly-
 GCC.TTC.AAC.CCC.GGG.ATG.GAG.GAT.ATT.CTT.GAC.TCT.GCA.ATG.GGC.
 Ala-Phe-Asn-Pro-Gly-Met-Glu-Asp-Ile-Leu-Asp-Ser-Ala-Met-Gly-
 ACT.AAT.TGG.GTC.CCA.GAA.GAA.GCC.TCT.GGA.GAG.GCC.AGT.GAG.ATT.
 Thr-Asn-Trp-Val-Pro-Glu-Glu-Ala-Ser-Gly-Glu-Ala-Ser-Glu-Ile-
 CCC.GTA.CCC.CAA.GGG.ACA.GAG.CTT.TCC.CCC.TCC.AGG.CCA.GGA.GGG.
 Pro-Val-Pro-Gln-Gly-Thr-Glu-Leu-Ser-Pro-Ser-Arg-Pro-Gly-Gly-
 GGC.AGC.ATG.CAG.ACA.GAG.CCC.GCC.AGA.CCC.AGC.AAC.TTC.CTC.TCA.
 Gly-Ser-Met-Gln-Thr-Glu-Pro-Ala-Arg-Pro-Ser-Asn-Phe-Leu-Ser-
 GCA.TCT.TCT.CCA.CTC.CCT.GCA.TCA.GCA.AAG.GGC.CAA.CAG.CCG.GCA.
 Ala-Ser-Ser-Pro-Leu-Pro-Ala-Ser-Ala-Lys-Gly-Gln-Gln-Pro-Ala-
 GAT.GTA.ACT.GGT.ACC.GCC.TTG.CCC.AGG.GTG.GGC.CCC.GTG.AGG.CCC.
 Asp-Val-Thr-Gly-Thr-Ala-Leu-Pro-Arg-Val-Gly-Pro-Val-Arg-Pro-
 ACT.GGC.CAG.GAC.TGG.AAT.CAC.ACC.CCC.CAG.AAG.ACA.GAC.CAT.CCA.
 Thr-Gly-Gln-Asp-Trp-Asn-His-Thr-Pro-Gln-Lys-Thr-Asp-His-Pro-
 TCT.GCC.CTG.CTC.AGA.GAC.CCC.CCG.GAG.CCA.GGC.TCT.CCC.AGG.ATC.
 Ser-Ala-Leu-Leu-Arg-Asp-Pro-Pro-Glu-Pro-Gly-Ser-Pro-Arg-Ile-
 TCA.TCA.CCG.CGC.CCC.CAG.GGC.CTC.AGC.AAC.CCC.TCC.ACC.CTC.TCT.
 Ser-Ser-Pro-Arg-Pro-Gln-Gly-Leu-Ser-Asn-Pro-Ser-Thr-Leu-Ser-
 GCT.CAG.CCA.CAG.CTT.TCC.AGA.AGC.CAC.TCC.TCG.GGC.AGC.GTG.CTG.
 Ala-Gln-Pro-Gln-Leu-Ser-Arg-Ser-His-Ser-Ser-Gly-Ser-Val-Leu-
 CCC.CTT.GGG.GAG.CTG.GAG.GGC.AGG.AGG.AGC.ACC.AGG.GAT.CGG.AGG.
 Pro-Leu-Gly-Glu-Leu-Glu-Gly-Arg-Arg-Ser-Thr-Arg-Asp-Arg-Arg-

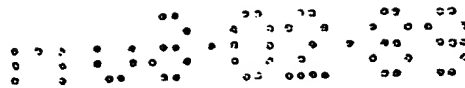


FIG. 5-3

AGC.CCC.GCA.GAG.CCA.GAA.GGA.GGA.CCA.GCA.AGT.GAA.GGG.GCA.GCC.
Ser-Pro-Ala-Glu-Pro-Glu-Gly-Gly-Pro-Ala-Ser-Glu-Gly-Ala-Ala-

AGG.CCC.CTG.CCC.CGT.TTT.AAC.TCC.GTT.CCT.TTG.ACT.GAC.ACA.GGC.
Arg-Pro-Leu-Pro-Arg-Phe-Asn-Ser-Val-Pro-Leu-Thr-Asp-Thr-Gly-

CAT.GAG.AGG.CAG.TCC.GAG.GGA.TCC.TCC.AGC.CCG.CAG.CTC.CAG.GAG.
His-Glu-Arg-Gln-Ser-Glu-Gly-Ser-Ser-Ser-Pro-Gln-Leu-Gln-Glu-

TCT.GTC.TTC.CAC.CTG.CTG.GTG.CCC.AGT.GTC.ATC.CTG.GTC.TTG.CTG.
Ser-Val-Phe-His-Leu-Leu-Val-Pro-Ser-Val-Ile-Leu-Val-Leu-Leu-

GCC.GTC.GGA.GGC.CTC.TTG.TTC.TAC.AGG.TGG.AGG.CGG.CGG.AGC.CAT.
Ala-Val-Gly-Gly-Leu-Leu-Phe-Tyr-Arg-Trp-Arg-Arg-Arg-Ser-His-

CAA.GAG.CCT.CAG.AGA.GCG.GAT.TCT.CCC.TTG.GAG.CAA.CCA.GAG.GGC.
Gln-Glu-Pro-Gln-Arg-Ala-Asp-Ser-Pro-Leu-Glu-Gln-Pro-Glu-Gly-

AGC.CCC.CTC.ACT.CAG.GAT.GAC.AGA.CAG.GTG.GAA.CTG.CCA.GTG.TAG.
Ser-Pro-Leu-Thr-Gln-Asp-Asp-Arg-Gln-Val-Glu-Leu-Pro-Val-***-

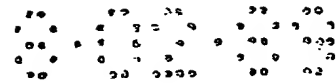


FIG. 6

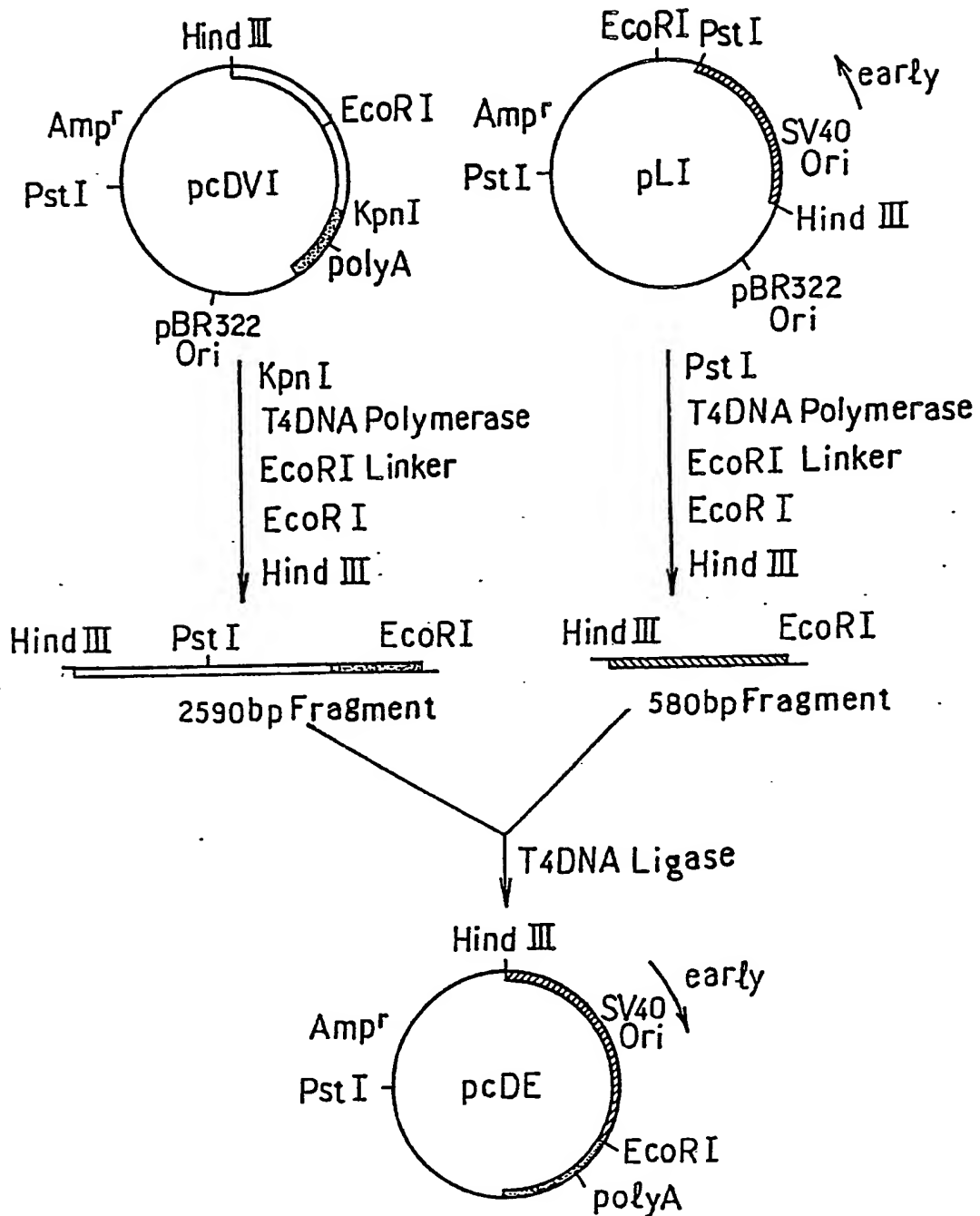


FIG. 7

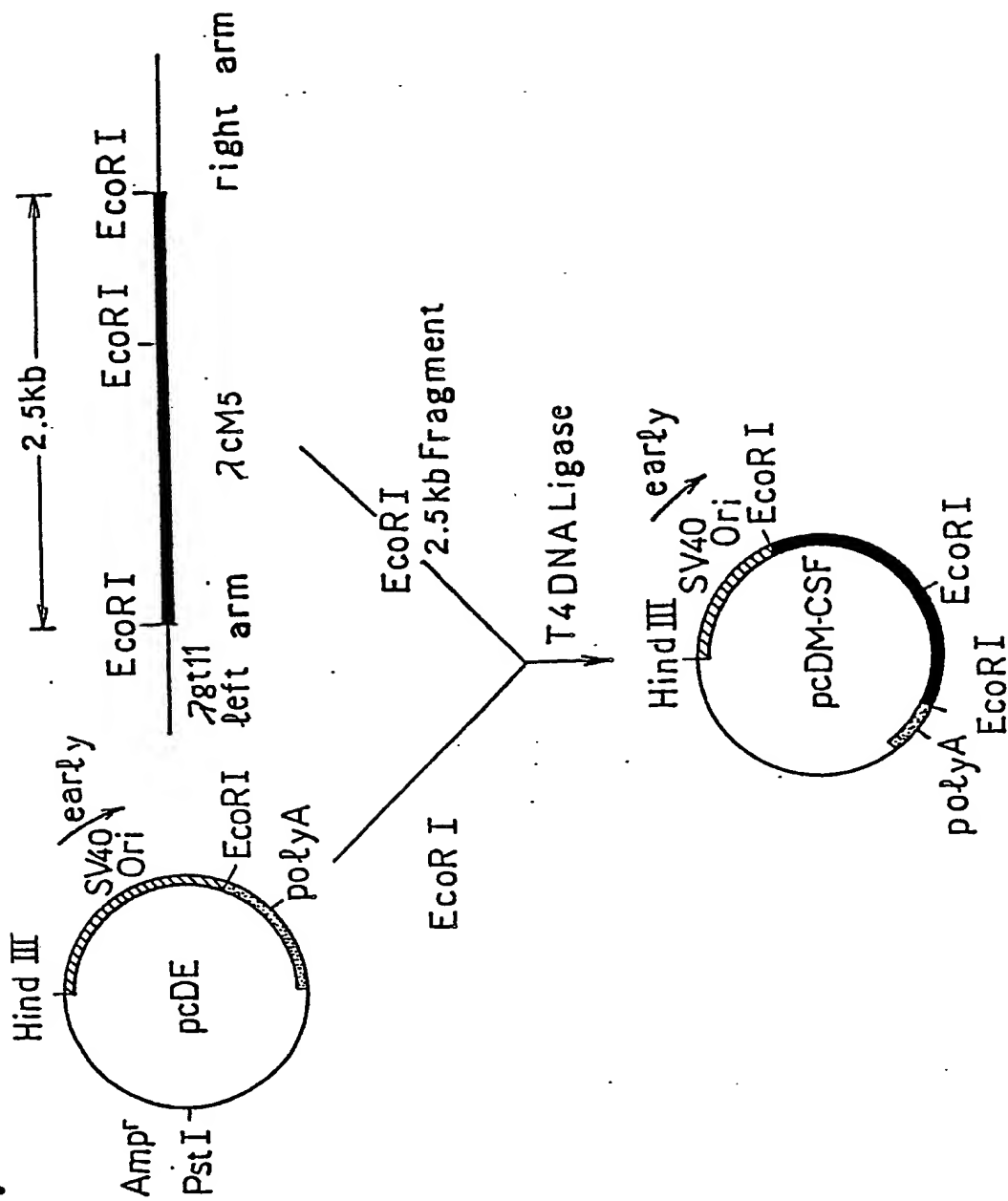
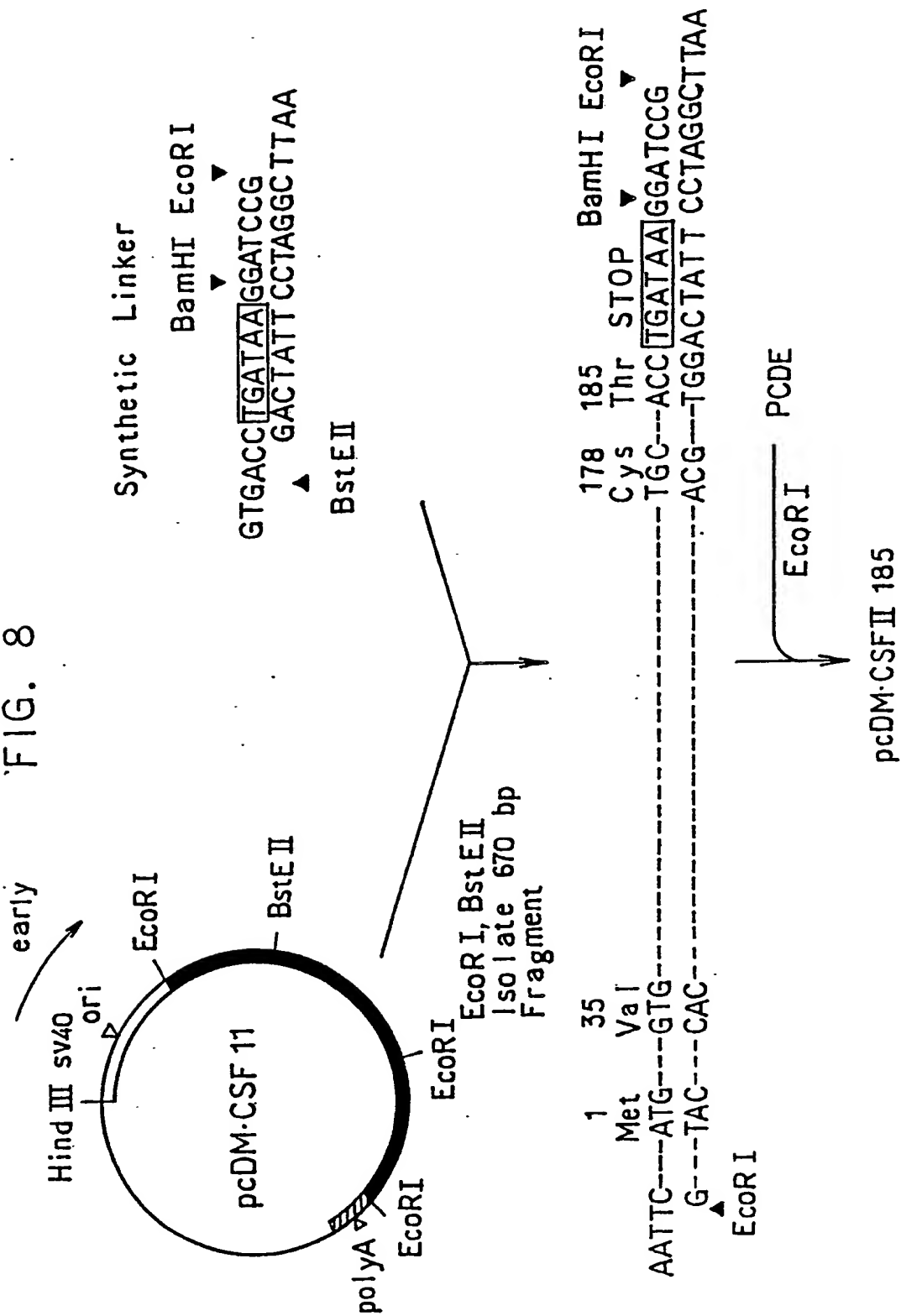
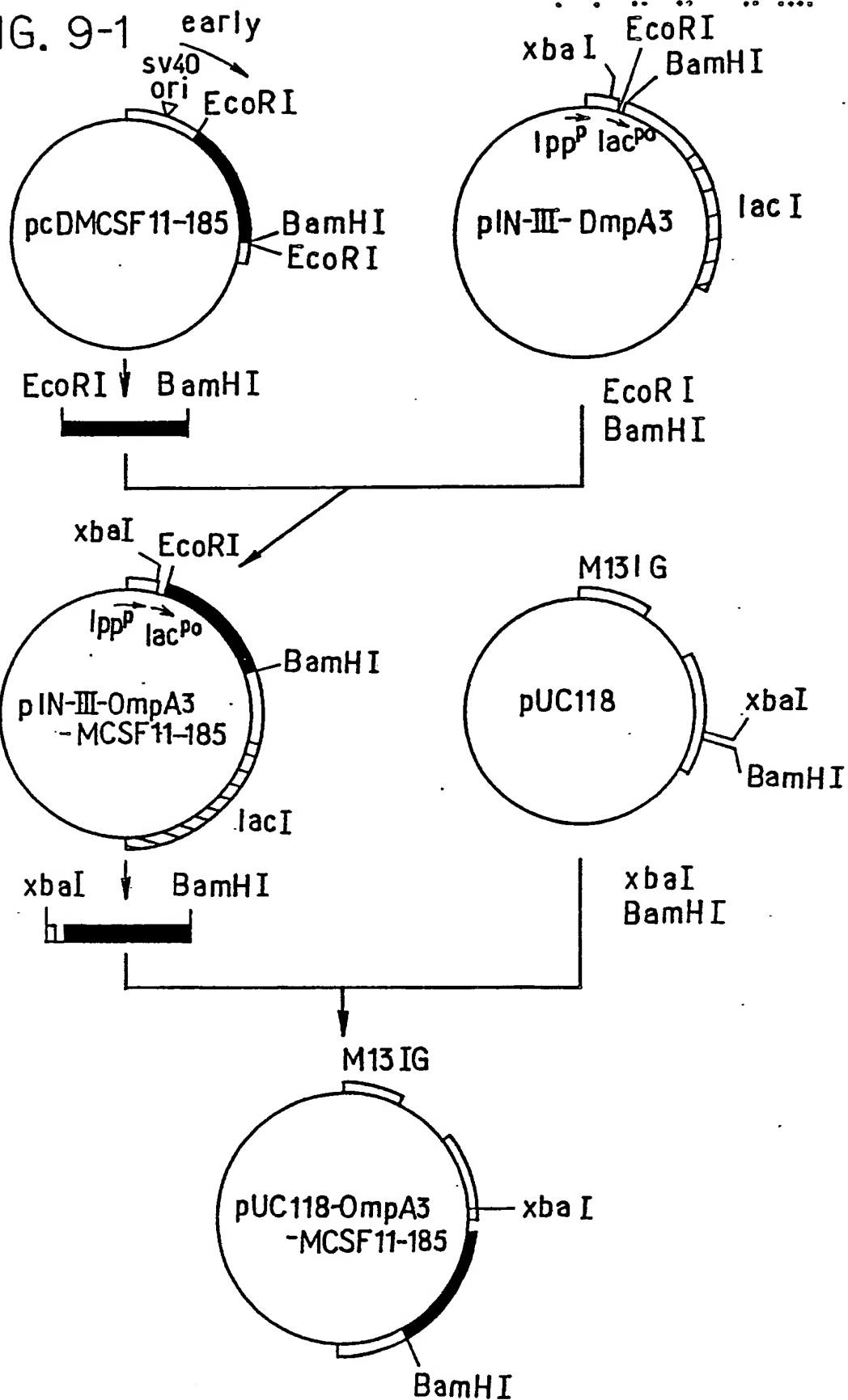


FIG. 8



early



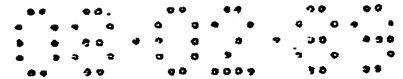


FIG. 9-2

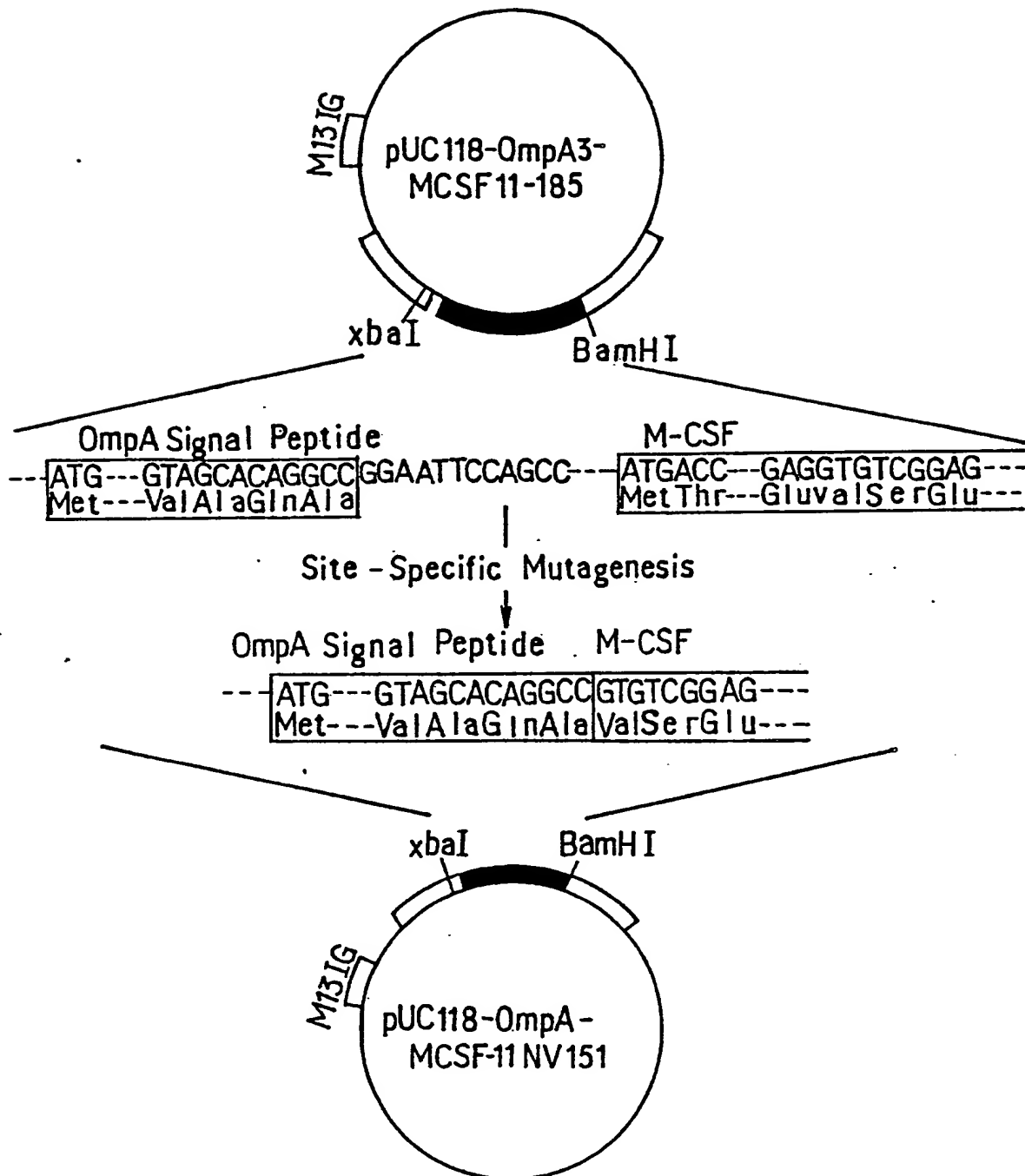
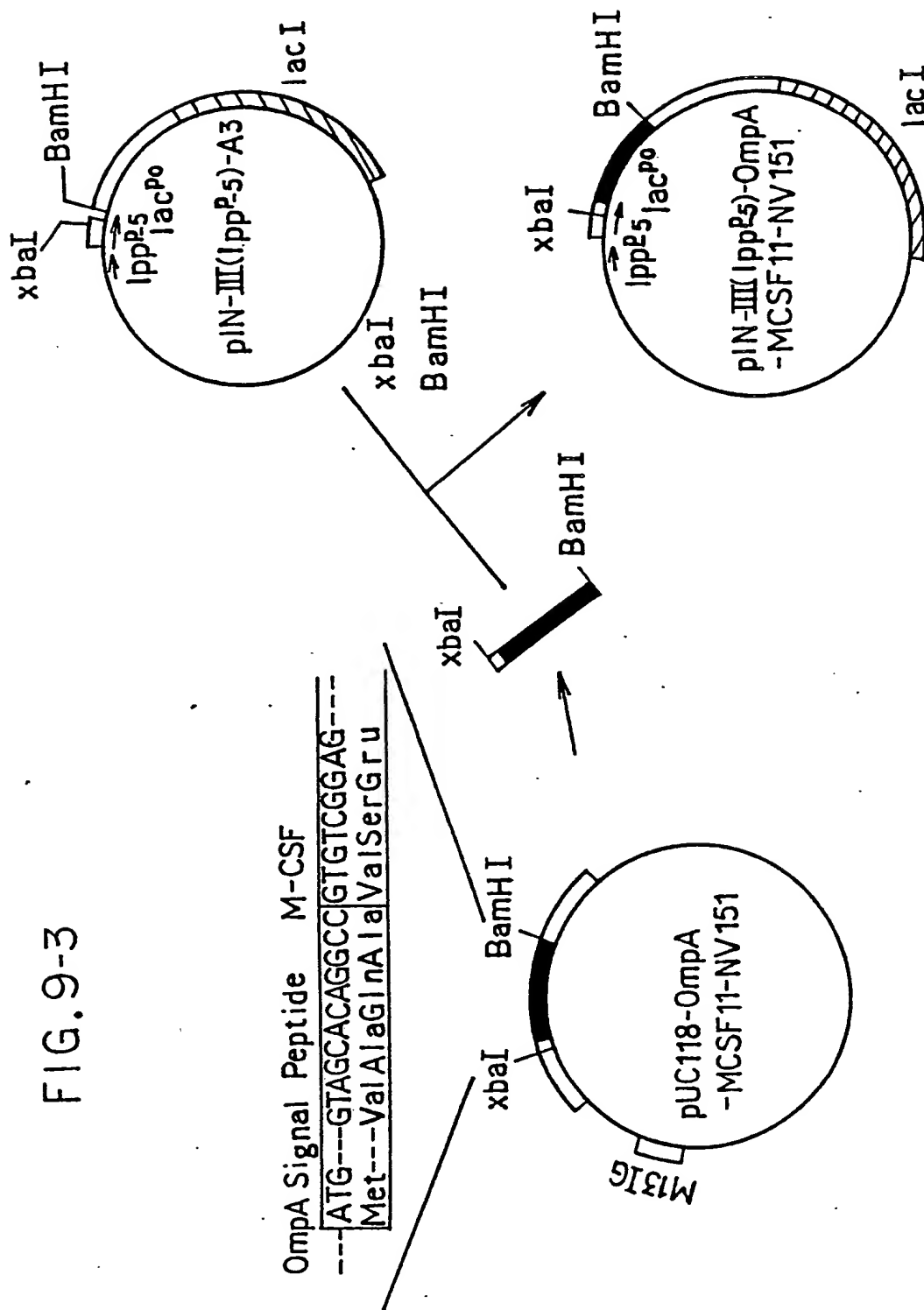


FIG. 9-3



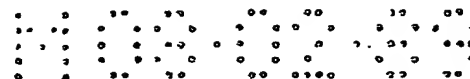


FIG. 10

Met-Lys-Lys-Thr-Ala-Ile-Ala-Ile-Ala-Val-Ala-Leu-Ala-Gly-Phe-
Ala-Thr-Val-Ala-Gln-Ala↓Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-
Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-
Met-Glu-Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-
Gln-Leu-Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-
Val-Gln-Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-
Pro-Asn-Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-
Leu-Lys-Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-
Cys-Val-Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-
Val-Lys-Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-
Trp-Asn-Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-
Ser-Ser-Gln-Asp-Val-Val-Thr

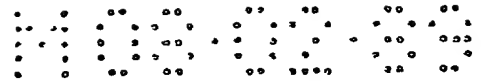
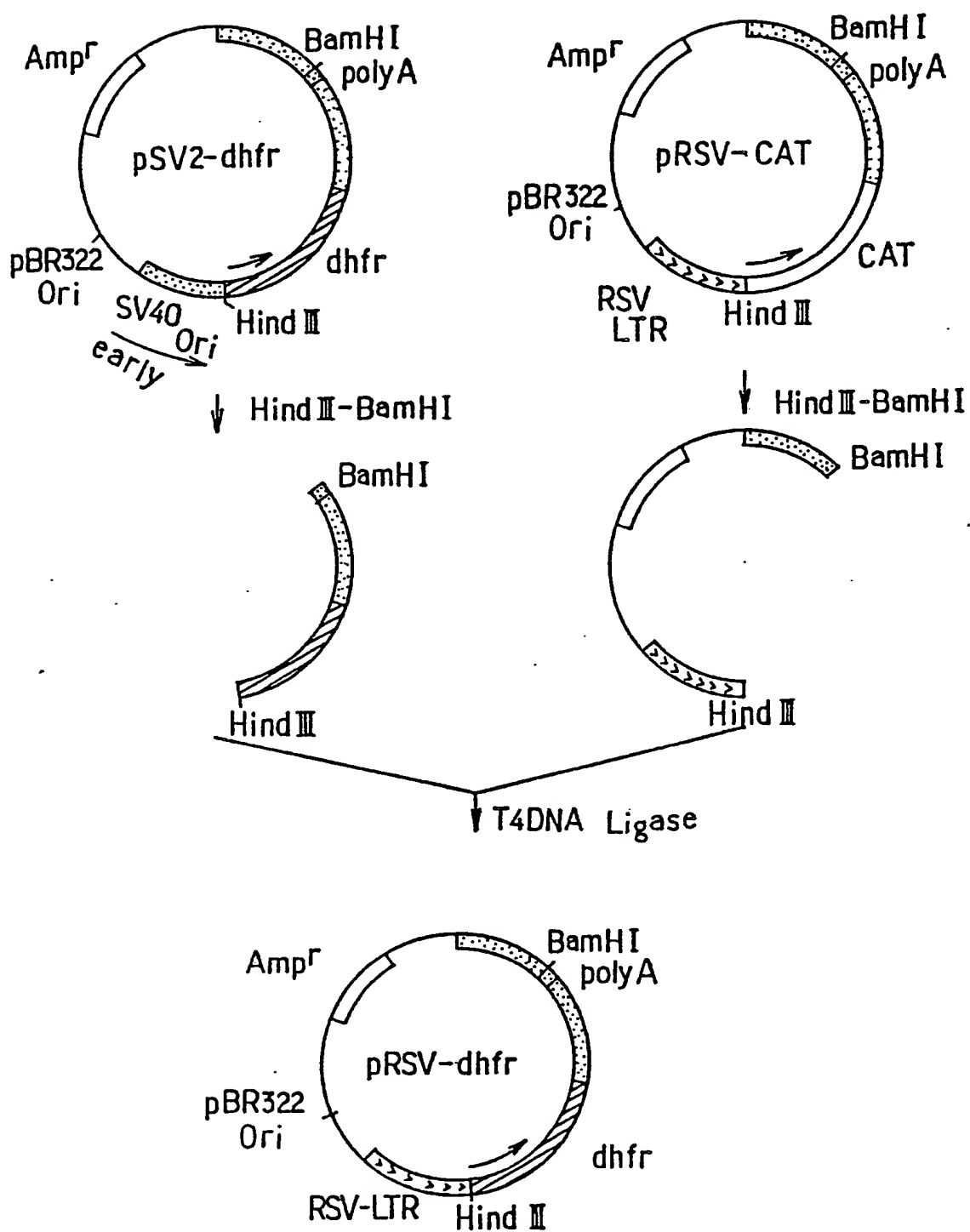


FIG. 11

Met-Lys-Lys-Thr-Ala-Ile-Ala-Ile-Ala-Val-Ala-Leu-Ala-Gly-Phe-
Ala-Thr-Val-Ala-Gln-Ala↓Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-
Gly-Ser-Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-
Met-Glu-Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-
Gln-Leu-Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-
Val-Gln-Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-
Pro-Asn-Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-
Leu-Lys-Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-
Cys-Val-Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-
Val-Lys-Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-
Trp-Asn-Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-

FIG. 12-1



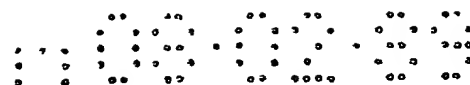
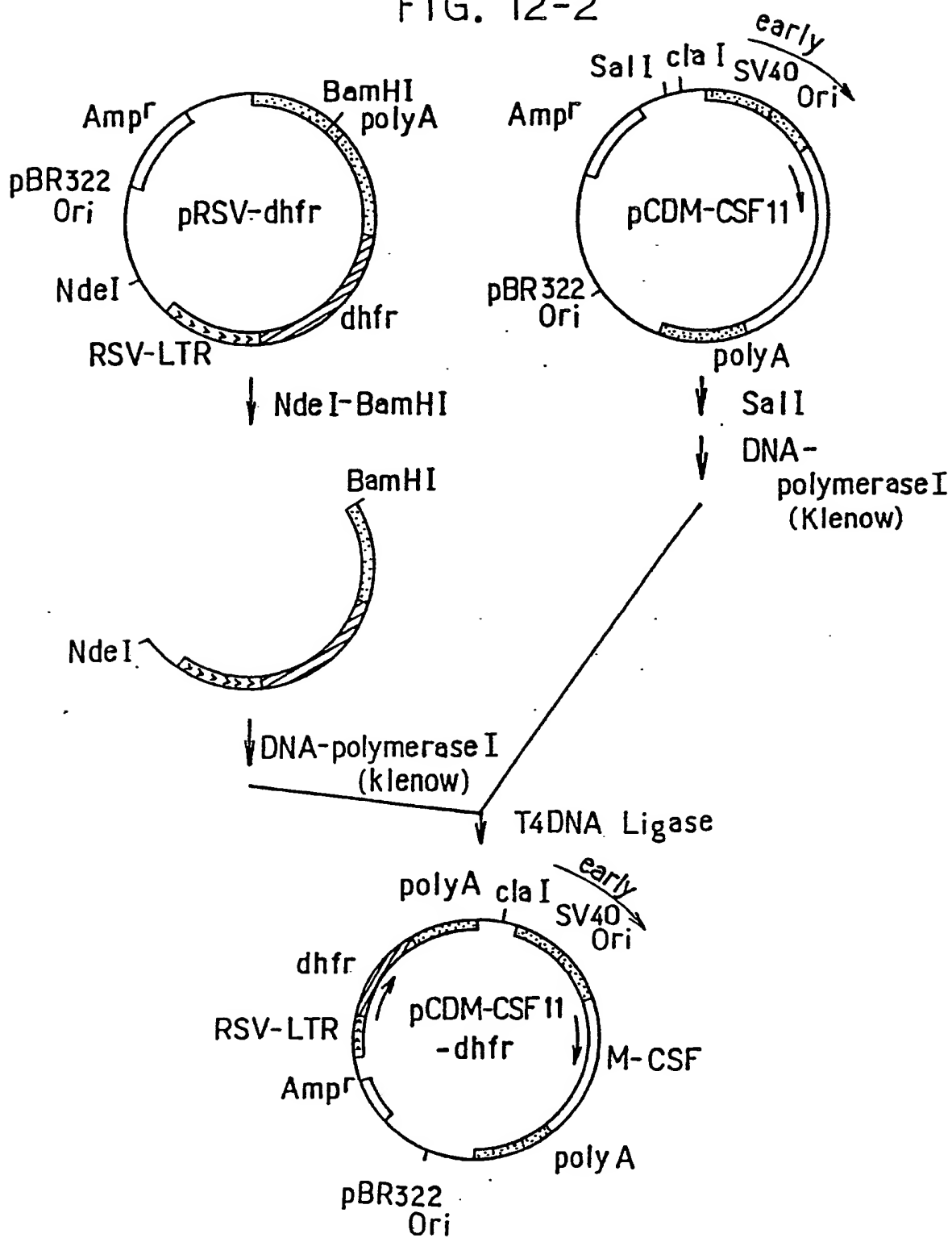


FIG. 12-2



1000000000

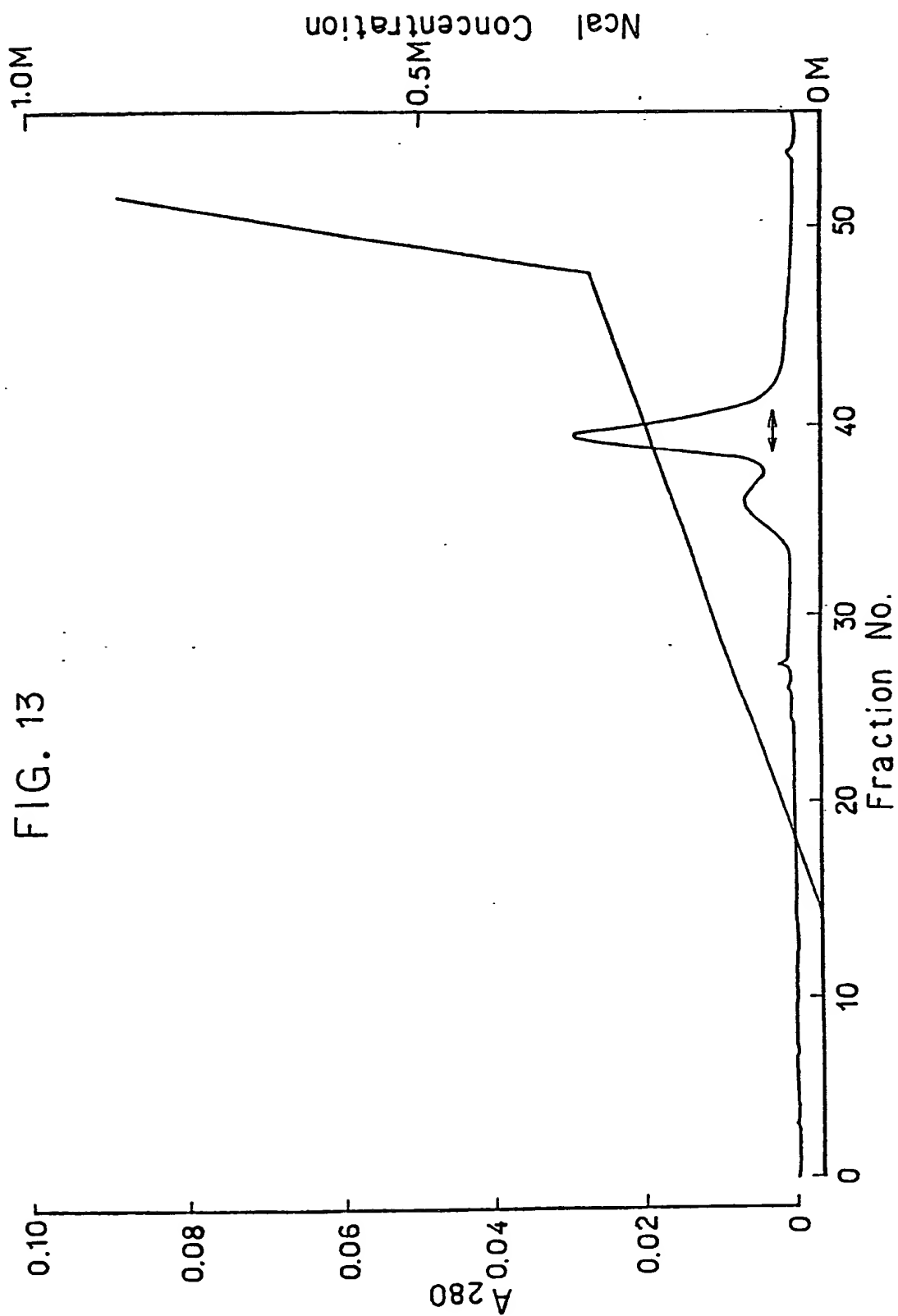
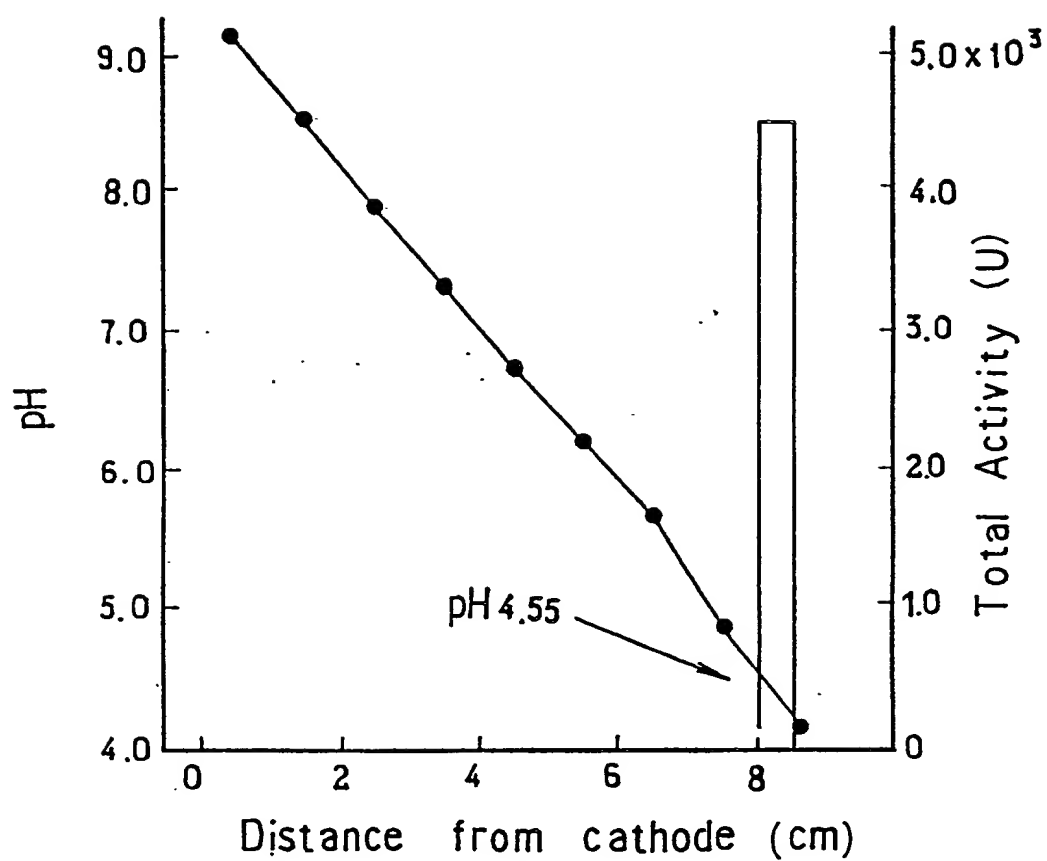


FIG. 13



FIG. 14



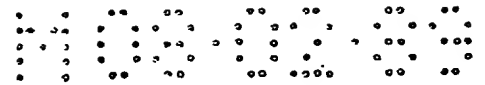


FIG. 15

ATG.GCA.CCT.
Met-Ala-Pro-

ACT.TCA.AGT.TCT.ACA.AAG.AAA.ACA.CAG.CTA.CAA.CTG.GAG.CAT.TTA.
Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-
IL-2

CTG.CTG.GAT.TTA.CAG.ATG.ATT.TTG.AAT.GGA.ATT.AAT.AAT.TAC.AAG.
Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-

AAT.CCC.AAA.CTC.ACC.AGG.ATG.CTC.ACA.TTT.AAG.TTT.TAC.ATG.CCC.
Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-

AAG.AAG.GCC.ACA.GAA.CTG.AAA.CAT.CTT.CAG.TGT.CTA.GAA.CGG.AGG.
Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Arg-Arg-
IL-2

ACT.CAT.TG ATG GTA.TCA.GAA.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
Thr-His ter Met-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
M-CSF

GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-

ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-

AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-

GAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-

GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys

AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-

CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-

AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-

ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-

CAA.GAT.GTG.GTG.ACC.TGA.TAA.
Gln-Asp-Val-Val-Thr ter ter
M-CSF

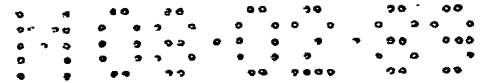


FIG. 16

ATG.GCA.CCT.
Met-Ala-Pro-
←

ACT.TCA.AGT.TCT.ACA.AAG.AAA.ACA.CAG.CTA.CAA.CTG.GAG.CAT.TTA.
Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-
IL-2

CTG.CTG.GAT.TTA.CAG.ATG.ATT.TTG.AAT.GGA.ATT.AAT.AAT.TAC.AAG.
Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-

AAT.CCC.AAA.CTC.ACC.AGG.ATG.CTC.ACA.TTT.AAG.TTT.TAC.ATG.CCC.
Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-

AAG.AAG.GCC.ACA.GAA.CTG.AAA.CAT.CTT.CAG.TGT.CTA.GAA.CGG.AGG.
Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Arg-Arg-
IL-2 →

ACT.TAA.TAA ATG GTA.TCA.GAA.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
Thr ter ter Met-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
← M-CSF

GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-

ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-

AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-

GAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-

GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys

AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-

CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-

AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-

ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-

CAA.GAT.GTG.GTG.ACC.TGA.TAA.
Gln-Asp-Val-Val-Thr ter ter
M-CSF →

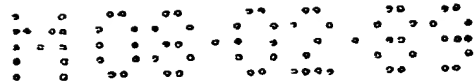


FIG. 17

ATG.GCA.CCT.ACT.TCA.
 Met-Ala-Pro-Thr-Ser-
 ← IL-2

AGT.TCT.ACA.AAG.AAA.ACA.CAG.CTA.CAA.CTG.GAG.CAT.TTA.CTG.CTG.
 Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-Leu-

GAT.TTA.CAG.ATG.ATT.TTG.AAT.GGA.ATT.AAT.AAT.TAC.AAG.AAT.CCC.
 Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-

AAA.CTC.ACC.AGG.ATG.CTC.ACA.TTT.AAG.TTT.TAC.ATG.CCC.AAG.AAG.
 Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-Lys-Lys-

GCC.ACA.GAA.CTG.AAA.CAT.CTT.CAG.TGT.CTA.GAA.CGG.AGG.ACT.CAT.
 Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Arg-Arg-Thr-His
 IL-2 →

TG ATG GAA.GAA.GTA.TCA.GAA.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
 ter Met-Glu-Glu-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
 ← M-CSF

GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
 Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-

ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
 Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-

AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
 Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-

GAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
 Asp-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-

GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
 Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys

AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
 Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-

CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
 Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-

AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
 Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-

ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
 Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-

CAA.GAT.GTG.GTG.ACC.TGA.TAA.
 Gln-Asp-Val-Val-Thr ter ter
 M-CSF →



FIG. 18

ATG.GCA.CCT.
Met-Ala-Pro-

ACT.TCA.AGT.TCT.ACA.AAG.AAA.ACA.CAG.CTA.CAA.CTG.GAG.CAT.TTA.
Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-
IL-2

CTG.CTG.GAT.TTA.CAG.ATG.ATT.TTG.AAT.GGA.ATT.AAT.AAT.TAC.AAG.
Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-

AAT.CCC.AAA.CTC.ACC.AGG.ATG.CTC.ACA.TTT.AAG.TTT.TAC.ATG.CCC.
Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-

AAG.AAG.GCC.ACA.GAA.CTG.AAA.CAT.CTT.CAG.TGT.CTA.GAA.CGG.AGG.
Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Arg-Arg-
IL-2 →

ACT.CAT.TG ATG GTA.TCA.GAA.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
Thr-His ter Met-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
← M-CSF

GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-

ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-

AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-

TAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
Tyr-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-

GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys

AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-

CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-

AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-

ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-

CAA.GAT.GTG.GTG.ACC.TGA.TAA.
Gln-Asp-Val-Val-Thr ter ter
M-CSF →

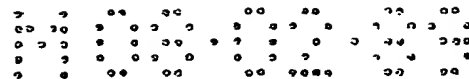


FIG. 19

ATG.GCA.CCT.
Met-Ala-Pro-
←

ACT.TCA.AGT.TCT.ACA.AAG.AAA.ACA.CAG.CTA.CAA.CTG.GAG.CAT.TTA.
Thr-Ser-Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-
IL-2

CTG.CTG.GAT.TTA.CAG.ATG.ATT.TTG.AAT.GGA.ATT.AAT.AAT.TAC.AAG.
Leu-Leu-Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-

AAT.CCC.AAA.CTC.ACC.AGG.ATG.CTC.ACA.TTT.AAG.TTT.TAC.ATG.CCC.
Asn-Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-

AAG.AAG.GCC.ACA.GAA.CTG.AAA.CAT.CTT.CAG.TGT.CTA.GAA.CGG.AGG.
Lys-Lys-Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Arg-Arg-
IL-2 →

ACT.TAA.TAA ATG GTA.TCA.GAA.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
Thr ter ter Met-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
← M-CSF

GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-

ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-

AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-

TAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
Tyr-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-

GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys

AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-

CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-

AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-

ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-

CAA.GAT.GTG.GTG.ACC.TGA.TAA.
Gln-Asp-Val-Val-Thr ter ter
M-CSF →

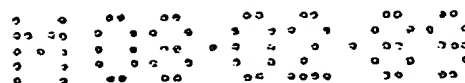


FIG. 20

ATG.GCA.CCT.ACT.TCA.
Met-Ala-Pro-Thr-Ser-
← IL-2

AGT.TCT.ACA.AAG.AAA.ACA.CAG.CTA.CAA.CTG.GAG.CAT.TTA.CTG.CTG.
Ser-Ser-Thr-Lys-Lys-Thr-Gln-Leu-Gln-Leu-Glu-His-Leu-Leu-Leu-

GAT.TTA.CAG.ATG.ATT.TTG.AAT.GGA.ATT.AAT.AAT.TAC.AAG.AAT.CCC.
Asp-Leu-Gln-Met-Ile-Leu-Asn-Gly-Ile-Asn-Asn-Tyr-Lys-Asn-Pro-

AAA.CTC.ACC.AGG.ATG.CTC.ACA.TTT.AAG.TTT.TAC.ATG.CCC.AAG.AAG.
Lys-Leu-Thr-Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr-Met-Pro-Lys-Lys-

GCC.ACA.GAA.CTG.AAA.CAT.CTT.CAG.TGT.CTA.GAA.CGG.AGG.ACT.CAT.
Ala-Thr-Glu-Leu-Lys-His-Leu-Gln-Cys-Leu-Glu-Arg-Arg-Thr-His
IL-2 →

TG ATG GAA.GAA.GTA.TCA.GAA.TAC.TGT.AGC.CAC.ATG.ATT.GGG.AGT.
ter Met-Glu-Glu-Val-Ser-Glu-Tyr-Cys-Ser-His-Met-Ile-Gly-Ser-
← M-CSF

GGA.CAC.CTG.CAG.TCT.CTG.CAG.CGG.CTG.ATT.GAC.AGT.CAG.ATG.GAG.
Gly-His-Leu-Gln-Ser-Leu-Gln-Arg-Leu-Ile-Asp-Ser-Gln-Met-Glu-

ACC.TCG.TGC.CAA.ATT.ACA.TTT.GAG.TTT.GTA.GAC.CAG.GAA.CAG.TTG.
Thr-Ser-Cys-Gln-Ile-Thr-Phe-Glu-Phe-Val-Asp-Gln-Glu-Gln-Leu-

AAA.GAT.CCA.GTG.TGC.TAC.CTT.AAG.AAG.GCA.TTT.CTC.CTG.GTA.CAA.
Lys-Asp-Pro-Val-Cys-Tyr-Leu-Lys-Lys-Ala-Phe-Leu-Leu-Val-Gln-

TAC.ATA.ATG.GAG.GAC.ACC.ATG.CGC.TTC.AGA.GAT.AAC.ACC.CCC.AAT.
Tyr-Ile-Met-Glu-Asp-Thr-Met-Arg-Phe-Arg-Asp-Asn-Thr-Pro-Asn-

GCC.ATC.GCC.ATT.GTG.CAG.CTG.CAG.GAA.CTC.TCT.TTG.AGG.CTG.AAG.
Ala-Ile-Ala-Ile-Val-Gln-Leu-Gln-Glu-Leu-Ser-Leu-Arg-Leu-Lys

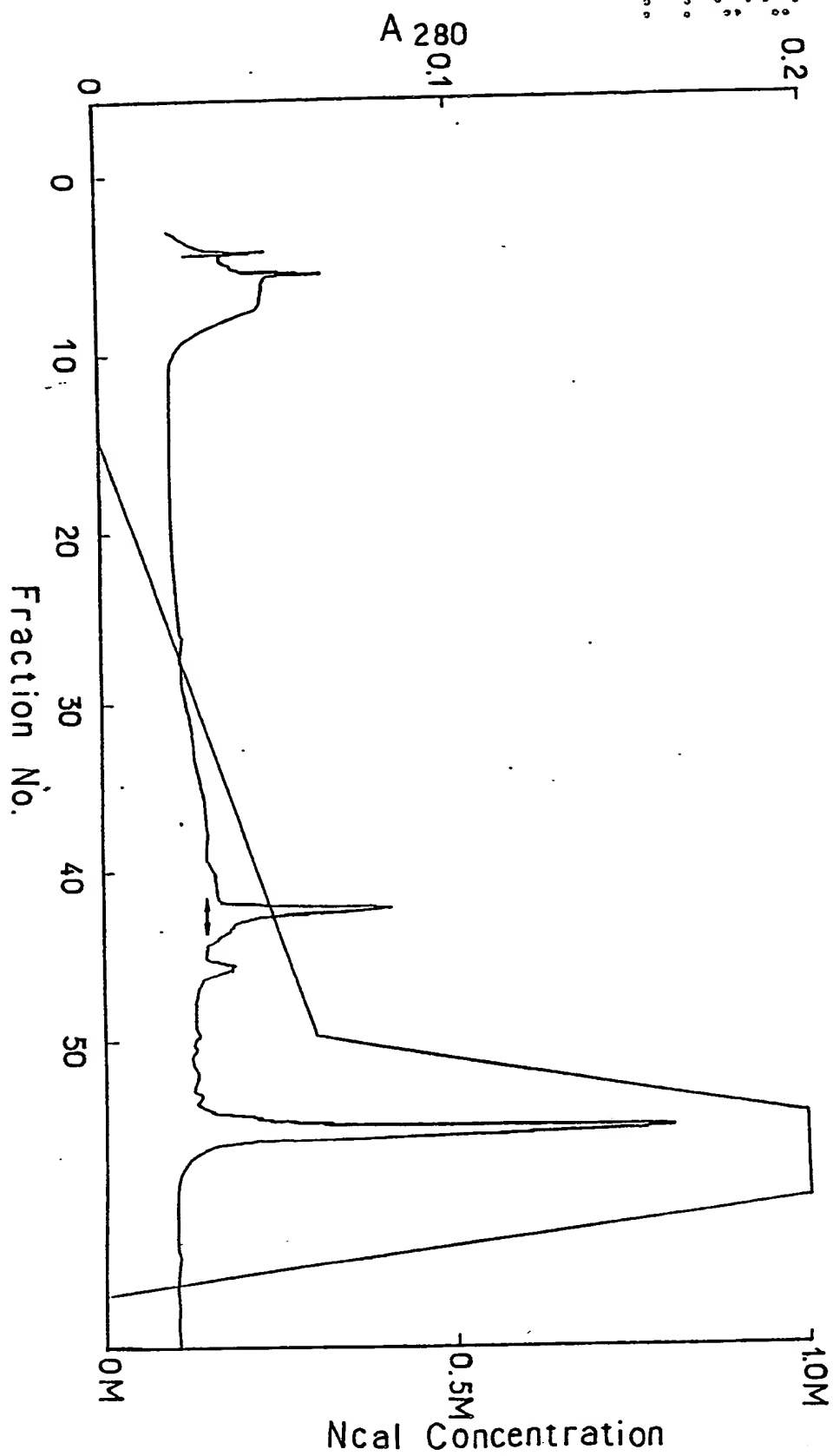
AGC.TGC.TTC.ACC.AAG.GAT.TAT.GAA.GAG.CAT.GAC.AAG.GCC.TGC.GTC.
Ser-Cys-Phe-Thr-Lys-Asp-Tyr-Glu-Glu-His-Asp-Lys-Ala-Cys-Val-

CGA.ACT.TTC.TAT.GAG.ACA.CCT.CTC.CAG.TTG.CTG.GAG.AAG.GTC.AAG.
Arg-Thr-Phe-Tyr-Glu-Thr-Pro-Leu-Gln-Leu-Leu-Glu-Lys-Val-Lys-

AAT.GTC.TTT.AAT.GAA.ACA.AAG.AAT.CTC.CTT.GAC.AAG.GAC.TGG.AAT.
Asn-Val-Phe-Asn-Glu-Thr-Lys-Asn-Leu-Leu-Asp-Lys-Asp-Trp-Asn-

ATT.TTC.AGC.AAG.AAC.TGC.AAC.AAC.AGC.TTT.GCT.GAA.TGC.TCC.AGC.
Ile-Phe-Ser-Lys-Asn-Cys-Asn-Asn-Ser-Phe-Ala-Glu-Cys-Ser-Ser-

CAA.GAT.GTG.GTG.ACC.TGA.TAA.
Gln-Asp-Val-Val-Thr ter ter
M-CSF →



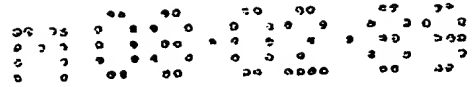


FIG. 22

